REGULATION OF A MULTIPOTENT SPINAL CORD PROGENITOR POPULATION

by

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STATEMENT OF DISSERTATION APPROVAL

The following faculty members served as the supervisory committee chair and members for the dissertation of Lisa Kristine Briona.

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ABSTRACT

Mammalian spinal cord injury is permanent due in part to a lack of local injury-induced neurogenesis. For the zebrafish, however, even complete spinal transection is temporary: the adult recovers within 6-8 weeks postinjury. We sought to create a companion model for studying regeneration after spinal cord injury (SCI) in the larval zebrafish. We have shown that recovery in the larval animal is even more rapid than in the adult: injured larvae exhibit sensory recovery within 2 days postinjury (dpi) and functional recovery by 5dpi, thereby establishing the larval zebrafish as a tractable companion model for studying regeneration after SCI.

Dbx1 expression labels a transient multipotent progenitor population in the murine spinal cord. As recovery from SCI relies in part upon a resident neural progenitor population, we asked whether an analogous population might exist beyond embryogenesis in the zebrafish. We found that *dbx1a*-expressing cells persisted as neurogenic radial glial progenitors beyond embryogenesis and that they contribute to the neurogenic response after SCI that may be exploited for healing measures after SCI.

We next asked if Wnt/β-catenin signaling might regulate SCI-induced regeneration. Using a Wnt reporter line, we found reporter expression in the blastema following injury, and that blastemal radial glia expressing the reporter were neurogenic. For further analysis, we generated a transgenic line with tamoxifen-inducible Cre activity to permanently label radial glia. During development, converted cells were quiescent spinal radial glial, nominally neurogenic. However, after SCI, converted cells proliferated in the blastema and became highly neurogenic. In the presence of IWR1, a

Wnt inhibitor, converted cells showed a neurogenic delay after SCI; this delay was due to inhibited expression of proneural gene ascl1a.

My work has characterized the contributions of radial glial neural progenitor cells during development and their injury-induced response during spinal cord regeneration. I've established new genetic tools and reproducible techniques for continued analysis of the mechanisms involved in spinal cord regeneration. Nature has already developed a regenerative solution to spinal cord injury; continued study of this amazing process will eventually lead to its recapitulation in regenerative medicine.

For my unflagging cheering section: Patricia and Paul O'Brien, Leigh-Anne Allison, and Michael Howe. Thanks for believing in me.

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CHAPTER 1

INTRODUCTION

Why we study spinal cord regeneration

The spinal cord acts as the conduit of the central nervous system (CNS), the bidirectional 'freeway' of information between the brain and rest of the body. In the event of spinal cord injury (SCI), that freeway is damaged: communication from the brain dead-ends at the level of injury, as does communication from the periphery. Some animals, like the zebrafish *Danio rerio* are able to recover sensory and motor function even after complete spinal transection (Becker et al., 2004). At this time, however, people are unable to recover lost abilities after SCI.

Spinal cord injury in humans: statistics

SCI is permanent in people, and affects multiple aspects of the afflicted's life: mobility, life expectancy, economic burden, and reduced quality of life (Strauss et al., 2006). An expensive chronic condition, SCI costs an average of $100k per year (FY 2013) in ongoing medical costs in the US (DeVivo, 2012). With approximately 12000 new cases of SCI in the US each year, continued research is necessary to identify therapeutic targets that could one day restore spinal function after injury (DeVivo, 2012; National Spinal Cord Injury Statistical Center, 2013).

Loss of sensory and motor function after SCI is permanent in humans

Several mechanisms have been identified that contribute to our inability to regenerate our spinal cord after injury including a lack of axonal growth at the injury site

(Bradbury et al., 2002), as well as the inability to reinitiate neurogenesis (Figure 1.1; Darian-Smith, 2009; Wang et al., 2014).

Inability to regenerate axons at site of injury

The ability to regrow severed axons through the site of injury is necessary to restore synaptic communication. However, in the first hours following spinal cord injury in humans, phagocytic macrophages at the injury site promote growth cone collapse and axonal dieback, possibly in an effort to contain the injury and prevent spread of infection (Busch et al., 2009; McPhail et al., 2004). Microtubule destabilization within the axon itself also promotes axonal retraction (Hellal et al., 2011).

The myelinating cells of the CNS are oligodendrocytes, which exist in symbiosis with neuronal axons. Axonal dieback promotes oligodendrocytic apoptosis. The loss of oligodendrocytes leads to additional axonal degeneration, which ultimately leads to a reduction in the number of surviving axons at the injury site (Stirling, 2004).

In addition to the loss of axons as a direct result of injury, there is collateral damage as well. Injured axons release their calcium stores into their microenvironment. This eflux of calcium triggers mass depolarization in uninjured axons, leading to degeneration of previously healthy neurons (Ouardouz et al., 2003; Stirling et al., 2014).

Lack of neurogenesis at site of injury

Local neurogenesis at the site of spinal injury is required for reestablishment of central pattern generators (CPGs), internal neural networks capable of supporting ongoing rhythmic movement such as walking, hopping, or swimming (MacKay-Lyons, 2002). Postembryonic neurogenesis is dependent upon a resident neural progenitor population (Emsley et al., 2005). Unfortunately, progenitor cell activation is extremely poor in the injured human spinal cord: spinal cord trauma recruits astrocytes to the injury site, where reactive astrogliosis forms a glial scar (Figure 1.2; Okada et al., 2004).

This glial scar is highly inhibitory towards neurogenesis: increased epidermal growth factor receptor (EGFR) expression in activated astrocytes triggers EGFR signaling in local progenitor cells at the injury site, promoting gliogenesis instead of differentiation along a neuronal pathway (Barkho et al., 2006; Ju et al., 2012).

Effects of SCI are not permanent in some animals

Unlike humans, multiple species of teleost fish and urodeles have been identified as retaining the ability to regenerate their spinal cord throughout the life of the animal (see Figure 1.3). Mammalian neonates born in an immature state such as opossums also temporarily retain the ability to repair their spinal cord after injury (Wheaton et al., 2011). However, it is not clear whether spinal cord repair in developmentally immature mammalian neonates is truly regeneration, an extension of the embryonic development program, or a combination of the two (Nicholls and Saunders, 1996).

Amniotes

Various spinal lesion methods including cervical spinal hemisection, thoracic spinal transection, and thoracic spinal crush have been used in rat, mouse, opossum, and cat neonates to study spinal cord repair (Bernstein-Goral and Bregman, 1993; Forssberg et al., 1980; Fry et al., 2003; Ujigo et al., 2014). Evidence for true regeneration in mammalian neonates primarily comes from double-labeling studies, where rostral axons are labeled pre-injury with a dye such as fast blue, and caudal axons are labeled postinjury with another dye such as rhodamine. In animals that regenerate, double-labeled axons are observed both in the injury site and rostral to the injury site. Double-labeled axons were found in postinjury rat, mouse, and opossum neonates, suggesting that these animals do indeed regenerate their spinal cords (Bates and Stelzner, 1993; Boulland et al., 2013; Saunders et al., 1998). However, this period of regeneration is

limited: opossums can regenerate their spinal cord providing the injury occurs before P12; injury after this timepoint is permanent. A similar timeframe is observed in rat and mouse as well (Nicholls and Saunders, 1996).

Anamniotes

The majority of spinal cord regeneration research in the anamniote has focused on the adult animal, using tail amputations, serial spinal transections, spinal hemisection, or spinal crush to induce injury (Becker et al., 1997; Egar and Singer, 1972; McHedlishvili et al., 2012; Moftah et al., 2008). Urodele amphibians, including the newt and axolotl, show robust axonal regrowth and neurogenesis even after serial spinal cord transection (Chernoff et al., 2003). Functional recovery in the injured adult urodele is observed as early as 4 weeks postinjury (Diaz Quiroz et al., 2014); however, sensory axons do not appear to regenerate (Zukor et al., 2011).

Unlike urodeles, regeneration after spinal cord injury includes both functional and sensory recovery in adult teleost fish. Carp and goldfish recovery approximates pre-injury levels within 60-100 days postinjury (Takeda et al., 2008; Yamada et al., 1995; Zottoli and Freemer, 2003), while the weakly electric ghost knifefish shows recovery within 22dpi (Unguez, 2013).

One teleost model of spinal cord regeneration in widespread use is the zebrafish *Danio rerio*. Spinal cord injury in the adult zebrafish has been well-characterized: axonal regrowth across the injury site begins within 3dpi (Goldshmit et al., 2012), and functional recovery is restored within 6-8wpi (Becker and Becker, 2008; Becker et al., 1997; Hui et al., 2010; Kuscha et al., 2012). To demonstrate that postinjury neurogenesis also occurs in the zebrafish, the Appel lab showed that olig2^{+} neural progenitor cells (NPCs) can (albeit rarely) generate motor neurons at the injury site 6-8 weeks postlesion (Reimer et al., 2008).

To be of maximal therapeutic benefit, however, spinal NPCs would have to show robust proliferation and neuronal differentiation following injury. In light of the limited neurogenic contribution of olig2^{+} NPCs following injury, other subpopulations of spinal NPCs would be ideal candidates for further investigation.

Neural progenitor cells during development

CNS neurons are derived from neuroepithelial progenitors, some of which differentiate as radial glia (Mori et al., 2005). During development, a subpopulation of these radial glia (RG) will persist as NPCs (Figure 1.4; Anthony et al., 2004; Malatesta et al., 2000). In the mammalian CNS, most NPCs lose their neurogenic ability during development, terminally differentiating as astrocytes by the end of embryogenesis (Hirabayashi and Gotoh, 2005; Miller and Gauthier, 2007; Rakic, 2003). However, in some anamniotes, RG do not terminally differentiate but instead persist widely throughout the CNS for the life of the animal (Schmidt et al., 2013; Tozzini et al., 2012). Intriguingly, just as in the embryonic mammalian CNS, some of the anamniotic RG persist as NPCs capable of reinitiating neurogenesis after injury (Kroehne et al., 2011; Rothenaigner et al., 2011). Identifying a population of spinal NPCs that persist beyond embryogenesis in a regenerative animal model is the first step in identifying a therapeutic target for spinal cord regeneration.

Postembryonic neurogenesis

RG NPCs persist in several previously identified stem cell niches in the CNS, including the sub-ventricular zone (SVZ) of the lateral ventricles, sub-granular zone (SGZ) of the hippocampus, the teleost telencephalon, and retina (Adolf et al., 2006; Ahmad et al., 2000; Cameron et al., 1993; Nagashima et al., 2013). NPCs in these microenvironments retain the ability to generate functional neurons that can be incorporated into existing neural architecture: for example, in the mammalian SVZ,

neuroblasts are continually generated that subsequently migrate to the olfactory bulb by way of the rostral migratory stream, where they differentiate as neurons (Lois and Alvarez-Buylla, 1994). Unlike the NPCs of the SGZ and SVZ, NPCs of the retina are generally quiescent beyond embryogenesis; however, in regenerative animals, they are able to respond to injury by reinitiating asymmetrical division that both maintains the NPC population and replaces damaged photoreceptors of the eye (Meyers et al., 2012).

Evidence for a stem cell niche in the spinal cord is minimal; however, there is a growing body of research suggesting that multipotent quiescent spinal NPCs persist in the ependymal zone of the central canal beyond embryogenesis (Hamilton et al., 2009; Ke et al., 2006; Ohori et al., 2006; Park et al., 2007; Sabourin et al., 2009; Yamamoto et al., 2001). Demonstrating that these quiescent spinal NPCs can re-enter the cell cycle and reinitiate neurogenesis in response to injury is important for future clinical applications focused on enhancing endogenous neuroregenerative modalities.

Use of stem cells in the treatment of spinal cord injury

Two main areas of research focus on spinal cord repair: axonal regrowth and neurogenesis via stem cell therapy (SCT). The general study of axonal regrowth can be further subdivided into mechanistic analysis of molecular inhibition and molecular activation. Studies of molecular inhibition of axonal regrowth following injury seek to find ways to "inhibit the inhibitor:" IL6R, Nogo-A, and EGFR are known inhibitors of axonal regeneration (Atalay et al., 2007; Ju et al., 2012; Okada et al., 2004). Physiologic or pharmacologic manipulation of the spinal injury microenvironment to repress these inhibitors would be of great benefit to the spinal regeneration field. Indeed, several promising agents have been identified and are currently in clinical trial: ozanezumab, which blocks Nogo expression; tocilizumab which inhibits IL6R expression; and dacomitinib, an inhibitor of EGFR (Emery et al., 2008; GlaxoSmithKline, 2000;

Massachusetts General Hospital et al., 2000). Identifying and enhancing the expression of pro-axonal regrowth molecules is equally important: musashi1, sodium hyaluronate-CNTF, microRNA-201, and FGF2 have each been implicated in promoting axonal regrowth following injury (Goldshmit et al., 2012; Okano, 2002; Ujigo et al., 2014; Wang et al., 2014). Additional research in this area is expected to be fruitful in the years to come.

SCT for SCI examines the application of transplant tissue or activation of endogenous NPCs. Pioneers in the SCT for SCI field began by transplanting fetal rat-derived segments of spinal cord into injured adult rats. These grafts successfully integrated into the adult spinal cord and made new neurons (Reier et al., 1983). In light of the ethical and moral considerations associated with embryonic SCT, some researchers have moved towards examining the efficacy of induced pluripotent stem cells (Nutt et al., 2013; Okano and Yamanaka, 2014; Sareen et al., 2014). While this field of research is still in its infancy, current results are encouraging: in most cases, transplanted cells are not rejected by the host and go on to make neurons, oligodendrocytes, and astrocytes. At this time, however, these newly formed neurons are not integrated into existing circuitry and do not contribute to functional recovery (Nutt et al., 2013).

An alternative SCT for SCI utilizes endogenous NPCs, and has much in common with the research focused on the molecular activation of axonal regrowth: both seek to identify molecules and pathways that can promote therapeutic improvement. Injection of granulocyte-macrophage colony stimulating factor (GM-CSF) immediately after spinal hemi-section inhibited glial scar formation in rats (Huang et al., 2009). In mice, GM-CSF injection immediately after SCI increased the number of NPCs at the site of injury and promoted a slight increase in the number of newly born neurons in the injury field at 14dpi; however, it is not known whether these new neurons were

integrated into existing circuitry or contribute to functional and sensory recovery (Hayashi et al., 2009).

Expression analysis of Sox11b mRNA after adult zebrafish SCI showed its upregulation in both the ependymal cells lining the central canal and in immature neurons (Guo et al., 2011). Using a vivo-morpholino (a morpholino designed for use in adult animals) against Sox11b, this same group showed that Sox11b is essential for functional recovery after spinal transection. Interestingly, their findings also suggest that the proneural transcription factor achaete-scute complex homolog 1a (Ascl1a/Mash1a) is a downstream target of Sox11b. Promising as these results are, more studies are required to clearly elucidate the role of Sox11b in adult neurogenesis after SCI, ideally using genetic tools.

The methods described for activating NPCs after SCI show some success in promoting neurogenesis at the site of injury. However, these methodologies use a shotgun approach: NPCs are being activated, but there is no way to identify which subpopulation(s) of NPCs are being targeted. Since some NPC populations preferentially differentiate as oligodendrocytes or astrocytes instead of neurons (Asano et al., 2009; Freeman, 2010; Steffenhagen et al., 2012), the ability to identify neural progenitor populations for activation would be helpful in moving these treatments into therapeutic application following SCI.

Identification of neural progenitors in the postembryonic spinal cord

New NPC populations can be identified using either a candidate approach via lineage analysis or discovery-based screening. Several NPC subpopulations have been identified using the candidate approach: Dbx1^{+} and olig2^{+} NPCs are discrete multipotent subpopulations, each able to contribute to all three spinal cell types in the mouse: oligodendrocyte, neuron, and astrocyte (Fogarty et al., 2005; Masahira et al., 2006; Park

et al., 2004). However, gene expression-mapping studies show gaps between the fields of these two NPC subpopulations, suggesting that there are other NPC subpopulations yet to be identified.

Database mining of known genes expressed in both the developing and adult spinal cord with enrichment criteria (i.e., the cells expressing these genes also have to express multiple radial glial markers such as GFAP and BLBP) has yielded markers of other NPC subpopulations (Petit et al., 2011). Microarray analysis of postSCI tissue and RNA-seq analysis of *Xenopus* regenerative vs. nonregenerative spinal cord have identified multiple candidate genes that may be involved in neurogenesis as well (Hui et al., 2014; Lee-Liu et al., 2014).

At the time I began my studies, these NPC markers had only been characterized in nonregenerative animals. Prior to identifying methods to reactivate spinal NPCs in humans, we first need to confirm expression of these NPC markers in a regenerative model, and then characterize and challenge their neurogenic capacity with SCI.

Role of Wnt signaling during development and injury repair

Wnts are secreted glycoproteins that play a critical role in determining cell polarity, body axis, and cell fate specification during development (Cadigan and Nusse, 1997; Logan and Nusse, 2004). Curiously, canonical Wnt signaling (β-catenin dependent Wnt signaling) is also upregulated by injury, regardless of tissue affected or type of injury (Lento et al., 2014; Ramachandran et al., 2011; Wang et al., 2011). The pathway begins with a Wnt ligand binding to target cell receptors and co-receptors Frizzled and LRP5/6, respectively. This event targets Axin2 to the cellular membrane, thereby facilitating the stabilization of cytoplasmic β-catenin, which can then move into the nucleus to activate transcription of Wnt target genes.

Role of Wnt signaling during spinal cord development

Several canonical Wnts are expressed during development of the dorsal spinal cord, including Wnt1, Wnt4, Wnt5a, Wnt8, and Wnt10. Wnts 1 and 5a govern corticospinal tract projection and correct pathfinding (Liu et al., 2005), while Wnt8 mediates the posteriorization of neural tissue (Erter et al., 2001). Wnts also regulate cell proliferation (Bonner et al., 2008; Zechner et al., 2003), and promote V2 neuronal differentiation while inhibiting motor neuron formation (Yu et al., 2008).

In mammals, canonical Wnt signaling is normally turned off by the end of embryogenesis. In contrast, animals with regenerative capacity as adults continue to express canonical Wnts at low levels in the spinal cord, expression of which is increased during the regenerative response to SCI (Caubit et al., 1997; Liu et al., 2008). However, the role of Wnt signaling after SCI has only been examined in its capacity to facilitate axonal regrowth – its role in injury-induced neurogenesis has not been examined.

Role of Wnt signaling in injury repair

Whether in bone, pancreas, skin, or CNS, Wnt signaling is upregulated following injury (Ito et al., 2007; Keefe et al., 2012; Meyers et al., 2012; Minear et al., 2010).

In response to zebrafish retinal injury, the terminally differentiated Müller glia (MG) dedifferentiate and re-enter the cell cycle, undergoing self-renewing proliferation to make multipotent progenitors that eventually replace injured photoreceptors (Figure 1.5A; Meyers et al., 2012). Genes associated with MG dedifferentiation include the proneural transcription factor Ascl1a and lin28, a protein associated with self-renewal of stem cells and regeneration (Ramachandran et al., 2011; Shyh-Chang et al., 2013). Ascl1a regulates the dedifferentiation program via canonical Wnt signaling: in the absence of Ascl1a, Dickkopf-related protein 1 (dkk1, an inhibitor of canonical Wnt signaling) is not suppressed, and fewer MG-derived photoreceptors are found in the retina postinjury

(Ramachandran et al., 2011). Additionally, inhibition of canonical Wnt signaling using genetic or pharmacologic means prevented injury-induced dedifferentiation of MG. When Wnt signaling was constitutively activated, dedifferentiated MG persisted as multipotent progenitors and failed to differentiate as either MG or photoreceptors (Meyers et al., 2012). Together, these findings suggest that canonical Wnt signaling is required for the dedifferentiation and proliferation of MG following injury, but not for their differentiation as photoreceptors.

Following caudal fin amputation, both dedifferentiated and local stem cells contribute to blastema formation in zebrafish (Figure 1.5B; Stoick-Cooper et al., 2007). Three stages of regeneration have been characterized following caudal fin amputation: formation of the wound epidermis is the first stage, and persists until 24 hours postinjury (hpi). Blastema formation is the second stage and lasts until 48hpi. The third stage, regenerative outgrowth, commences at 48hpi and persists until healing is complete. Wnt expression is detectable at the distal tip of the tail with 3hpi, with Wnt reporter TOPdGFP expression detectable in the blastema by 2dpi (Stoick-Cooper et al., 2007). To verify that canonical Wnt signaling was required for caudal regeneration, the Moon lab injured *hs:dkk1-GFP* fish and subjected them to heat shock during recovery.

They found that if fish were heat-shocked beginning at 24hpi, no regeneration occurred. Further analysis showed that the block in regeneration was not due to failed wound healing; rather, it was due to failure of blastemal formation and proliferation. If instead fish were heat-shocked beginning at 72hpi, regeneration was arrested due to lack of cell differentiation. Conversely, overexpression of Wnt using *hs:wnt8a-GFP* showed enhanced regeneration and increased proliferation of the blastema (Stoick-Cooper et al., 2007). These data indicate that canonical Wnt signaling is required for the proliferative response to caudal fin amputation, as is also observed in the regenerating retina. These

data also show a role for canonical Wnt signaling in differentiation after caudal fin amputation, which is not required in the regenerating retina.

The role of Wnt signaling in SCI-induced neurogenesis has not been previously characterized. However, preliminary studies using *hs:dkk1-GFP* fish treated with BrdU from 4-5 days postfertilization (dpf), injured at 5dpf, and heat-shocked twice daily during recovery showed a significant reduction in both the number of $BrdU^+$ cells and the number of newly born neurons in the blastema, suggesting a requirement for canonical Wnt signaling in spinal cord regeneration as well. Identifying a mechanism by which the neurogenic response to SCI is regulated will help focus ongoing research in spinal cord regeneration.

Overview of Chapters 2-4

Chapter 2 presents the larval model of spinal cord injury utilized in Chapters 3 and 4. A companion model to the adult zebrafish, this paradigm takes advantage of speedy throughput, faster recovery times, larval transparency for *in vivo* observations, and the availability of genetic models not otherwise accessible in the adult.

Chapter 3 focuses on characterizing an NPC population identified via *dbx1a* expression. I first demonstrated that *dbx1a* mRNA was expressed until at least 14dpf, and confirmed that in the *dbx1a:eGFP* line, *GFP* and *dbx1a* mRNAs co-localized in the spinal cord. I next showed that *dbx1a:eGFP*$^+$ progenitors persist beyond embryogenesis as $GFAP^+$ radial glia that contribute to the spinal neuronal population. I also verified that *dbx1a:eGFP*$^+$ NPCs were a separate subpopulation from *olig2*$^+$ NPCs, and that more *dbx1a:eGFP*$^+$ NPCs expressed the neural progenitor marker Sox3 than their *olig2*$^+$ counterparts at 5dpf. Using *Tg(elavl3:eGFP)* to label all neurons in the spinal cord, I established the timeline of recovery following SCI at 5dpf: extensions from the rostral stump projecting into the injury field were detected as early as 5dpi, with functional and

sensory recovery by 9dpi. Lastly, I showed that *dbx1a:eGFP*$^{+}$ cells proliferate and make new neurons in response to injury.

Chapter 4 examines the role of Wnt signaling following SCI using the injury model described in Chapter 2. Using the Wnt reporter *Tg(7xTCF-Xia.Siam:GFP)*, I first confirmed that Wnt was expressed in the blastema from 1-7dpi. Using this same line, I next demonstrated that GFP^{+} cells are radial glial neural progenitors (GFAP^{+}, Sox3^{+}) and that these GFP^{+} cells can make neurons. To continue our analysis, I chose to pharmacologically inhibit Wnt signaling using IWR1. To confirm that IWR1 has no effect on endogenous homeostasis, I incubated uninjured fish in IWR1 starting at 5dpf for 2 or 5 days. I showed that IWR1 does not affect homeostatic rates of proliferation in the gut (a tissue with a high rate of turnover) and the spinal cord (low rate of turnover) after long or short pulses of IWR1. I next introduced a new line generated to label spinal radial glia with temporal control: *Tg(GFAP:Cre^{ERt2}, cry:YFP)*. Crossing this line to *Tg(ubi:loxP-GFP-stop-loxP-mCherry) ("Ubi:switch")*, administering 4-OHT (a tamoxifen metabolite), and subsequent characterization of converted red cells showed that they colocalized with GFAP antibody staining, confirming this was an effective tool for permanently labeling radial glia. Additional analysis showed that developmentally, red cells do make neurons, albeit rarely; I also showed that converted cells were typically quiescent, with few BrdU^{+},dsRed^{+} cells observed in the intact spinal cord. To determine the response of converted cells to SCI, I injured animals at 5dpf and incubated them with or without IWR1. I found that converted cells arrested as GFAP^{+} progenitors rather than differentiating as neurons. To explain this observation, I confirmed that inhibition of canonical Wnt signaling after SCI using IWR1 reduced both the number of red BrdU^{+} cells in the blastemal as well as the number of converted neurons.

Taken together, I have established both a new model of spinal cord injury to study the molecular mechanisms of regeneration, and a new genetic tool to permanently

label radial glia under temporal control. I have showed that NPCs in the larval zebrafish spinal cord robustly respond to injury by proliferating and increasing their rate of neurogenesis. I have also demonstrated the requirement for canonical Wnt signaling in regenerative neurogenesis, which is explained in detail in Chapters 2-4.

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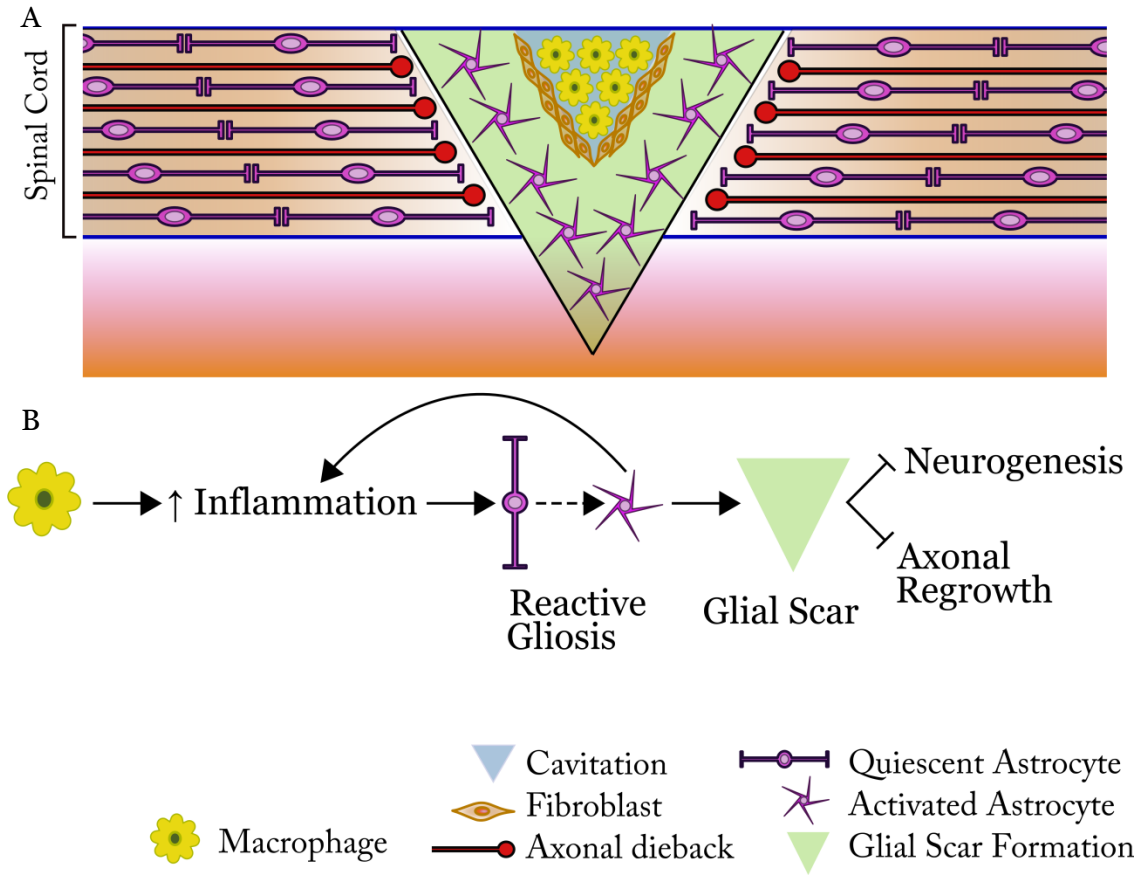

Figure 1.1: Spinal cord injury in nonregenerative animals. (A) Injured spinal cord. (B) Macrophage-induced inflammation triggers reactive gliosis in local astrocytes, leading to the formation of a glial scar. Both the microenvironment and secreted factors of the glial scar inhibit axonal regrowth and neurogenesis, resulting in permanent injury. Adapted from Silver and Miller, 2004.

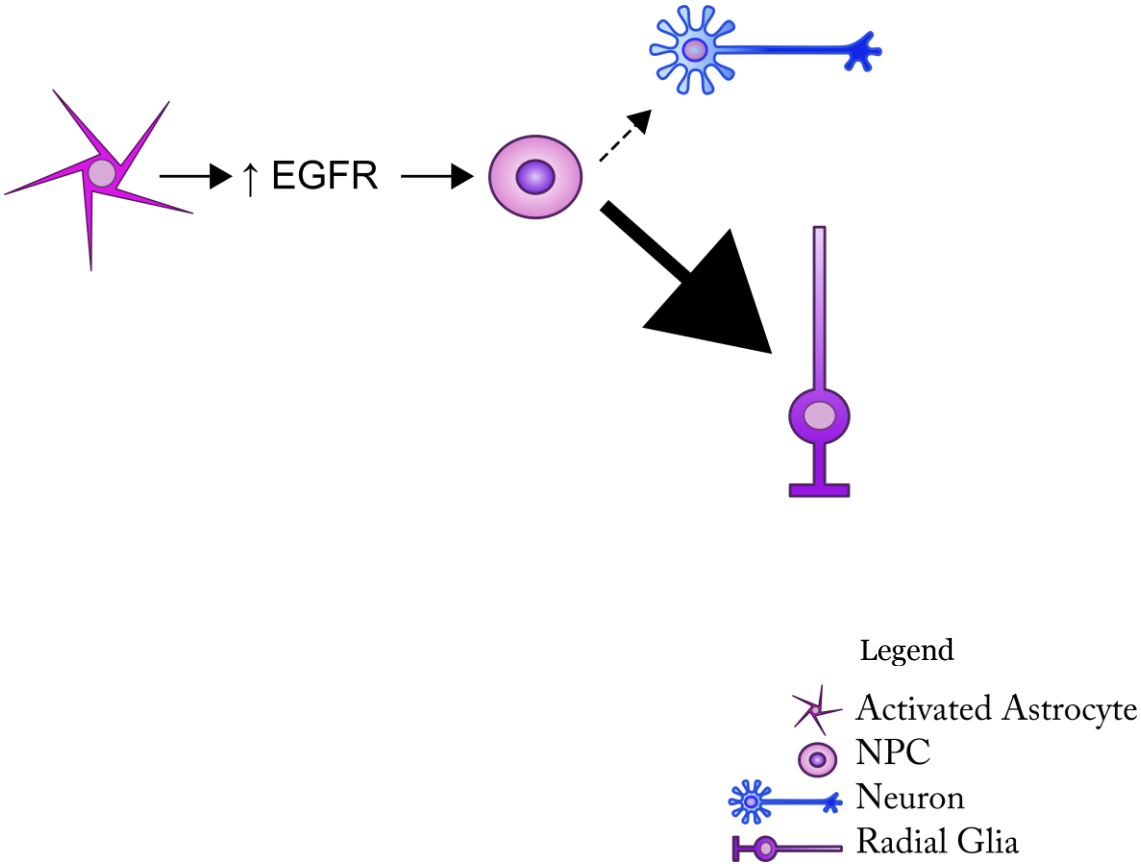

Figure 1.2: Glial scar inhibits injury-induced neurogenesis. Activated astrocytes in the glial scar express high levels of EGFR, triggering EGFR signaling in NPCs, promoting differentiation as radial glia instead of neurons.

Figure 1.3: Spinal cord injury in regenerative animals. (A) uninjured spinal cord. (B) Within 24 hours postinjury in the larval zebrafish (1 week postinjury in the newt), spinal stumps have retracted from plane of injury and a regenerative blastema has formed. (C) Between 1-3 days postinjury in the larval zebrafish (1-6wpi in newt), radial glia extend processes into injury site from both stumps; NPCs and newly born neurons are detectable in the blastema by this time, as are regrowing axons. (D) By 9dpi in larval zebrafish (6-8wpi in adult zebrafish, 6-9wpi in newt), the stumps have rejoined and injured animals exhibit functional recovery. CC = central canal. Adapted from Zukor, Kent & Odelberg 2011.

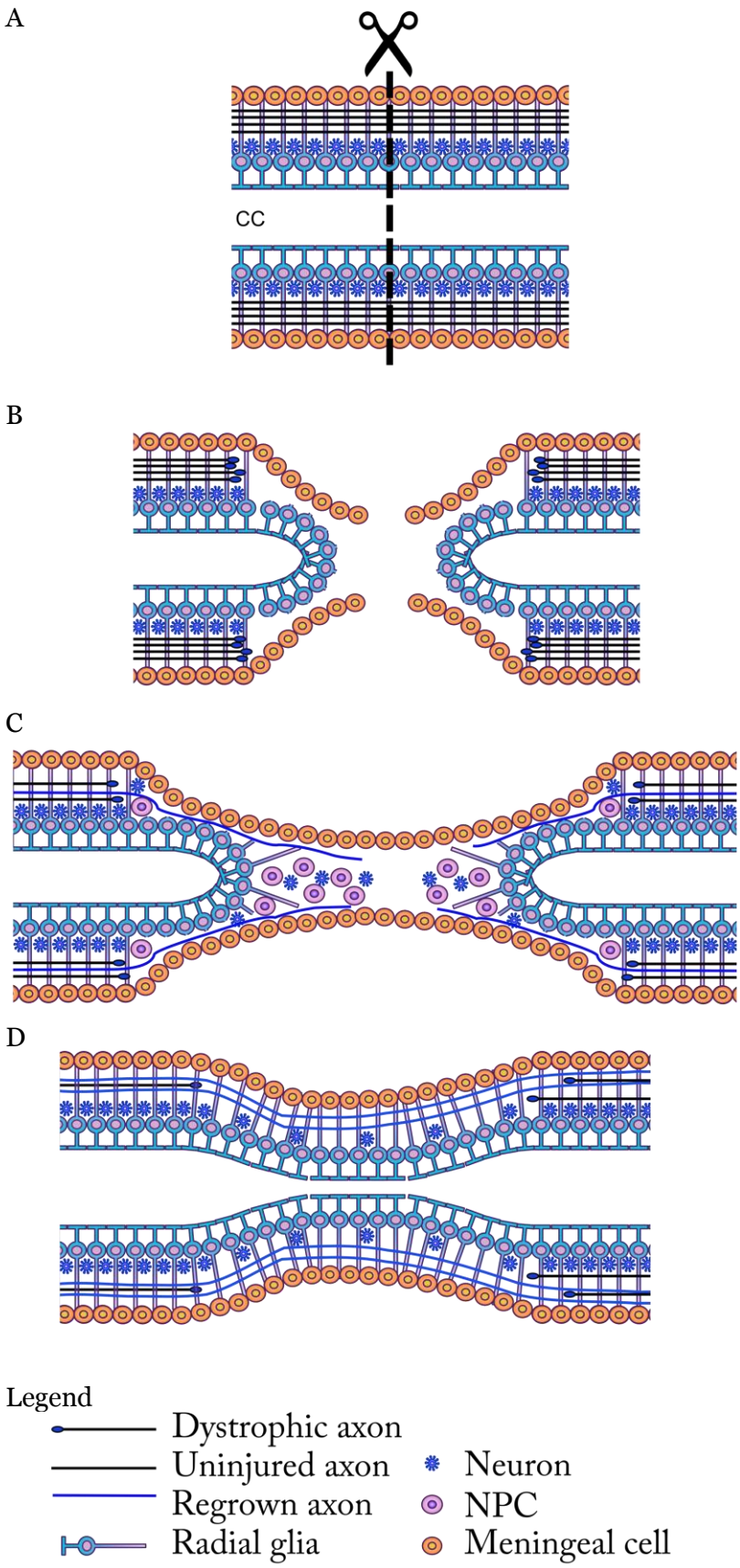
A
CC
B
C
D
Legend
Dystrophic axon
Uninjured axon
Regrown axon
Radial glia
Neuron
NPC
Meningeal cell

Figure 1.3

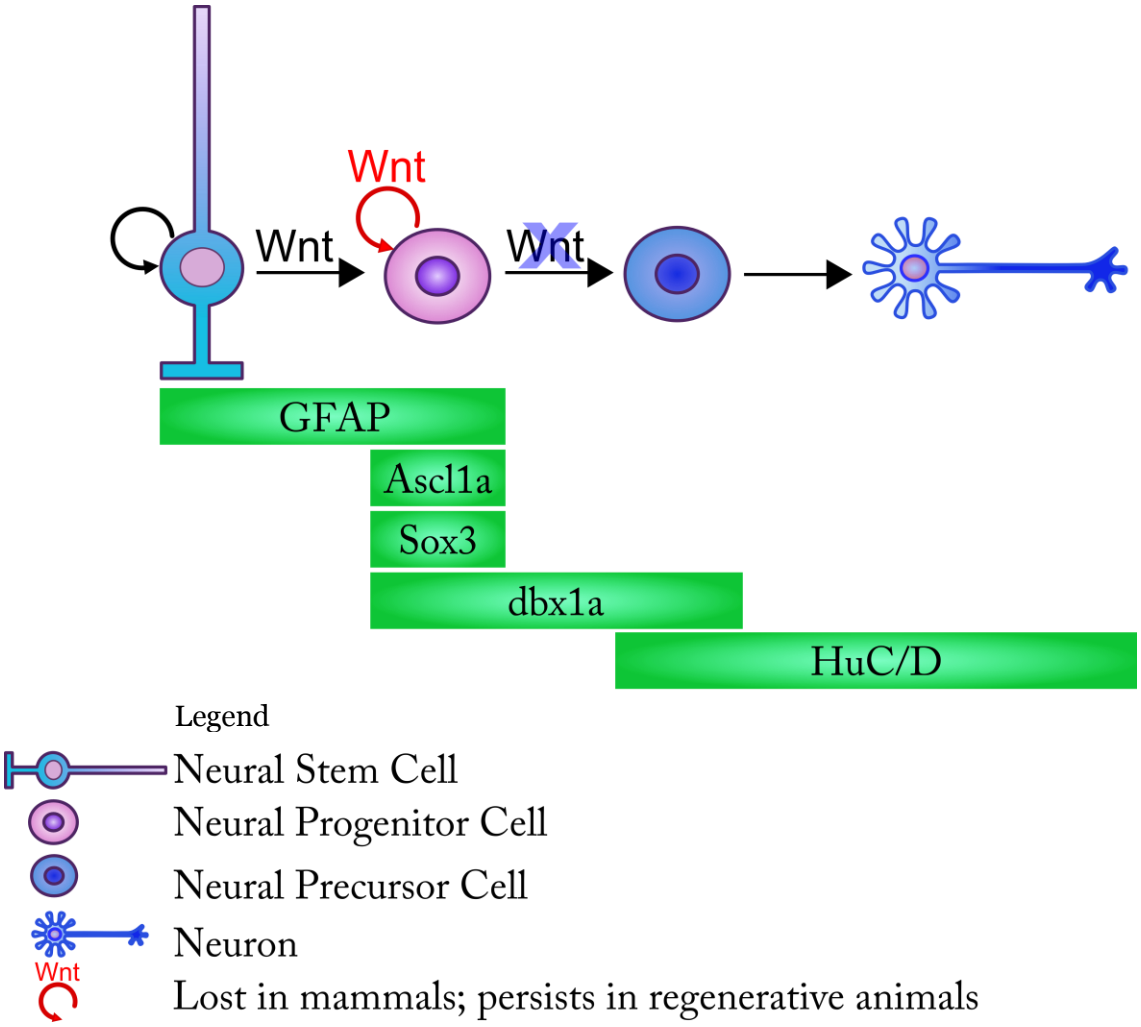

Figure 1.4: Neural development. Radial glial marker GFAP is expressed by both neural stem cells (NSCs) and NPCs. Wnt is required for the differentiation of NSC to NPC, but must be inhibited for an NPC to progress to neural precursor. In regenerative animals, NPC self-renewal persists beyond embryogenesis due to low levels of continued canonical Wnt expression; this self-renewal capacity is lost neonatally in non-regenerative animals. Various markers identify the progression of NSC to neuron.

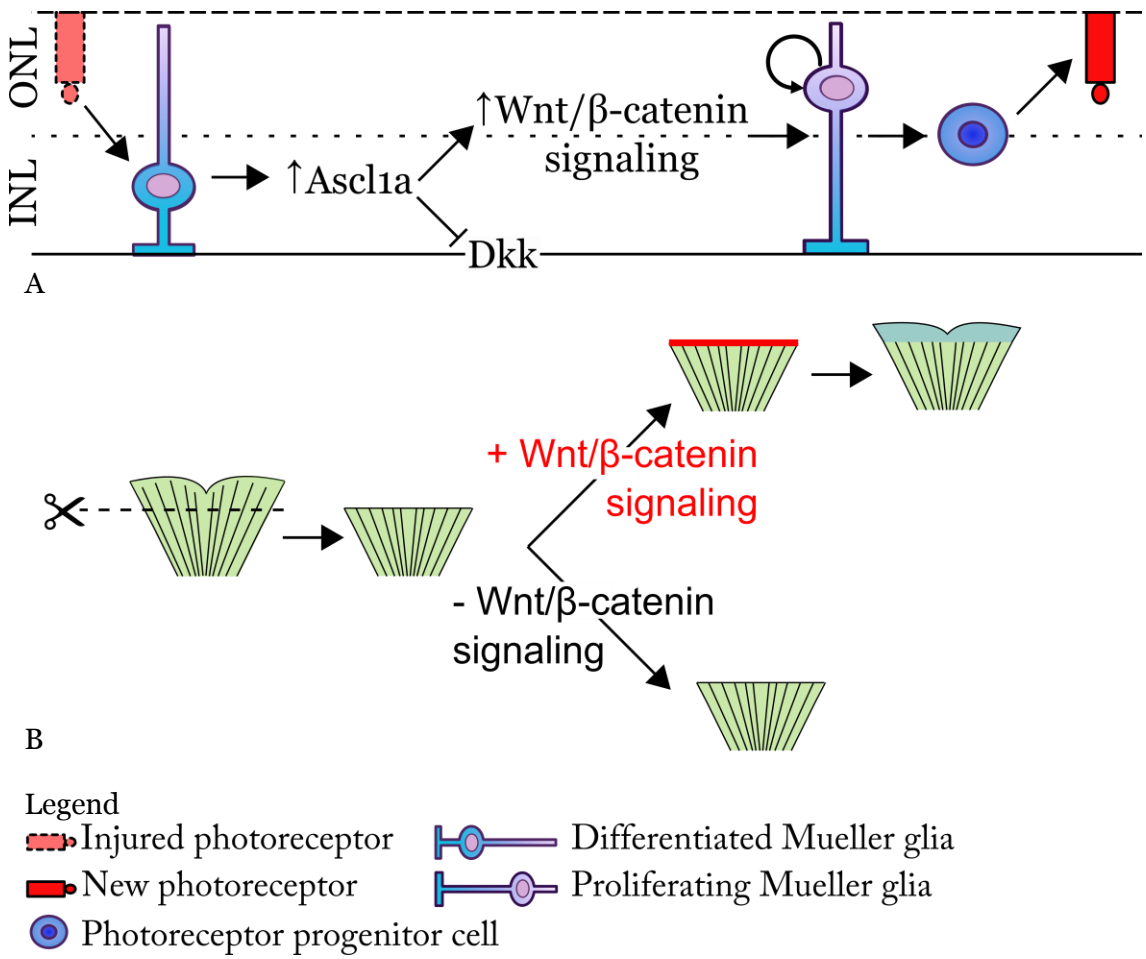

Figure 1.5: Wnt in zebrafish injury repair. (A) In the regenerating retina, damaged photoreceptors trigger the activation of quiescent Müeller glia to resume proliferation and the production of new photoreceptors. Mueller glia increase expression of Ascl1a which promotes Wnt/β-catenin signaling by inhibiting Dkk. (B) After caudal fin amputation, Wnt/β-catenin signaling is detected at the distal tip within 2dpi. In the absence of canonical Wnt signaling, no blastema forms, and no regeneration is observed.

CHAPTER 2

SPINAL CORD TRANSECTION IN THE LARVAL ZEBRAFISH

Reprint of: Briona, L. K., Dorsky, R. I. Spinal Cord Transection in the Larval Zebrafish. J. Vis. Exp. (87), e51479, doi:10.3791/51479 (2014).

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Video Article

Spinal Cord Transection in the Larval Zebrafish

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Abstract

Mammals fail in sensory and motor recovery following spinal cord injury due to lack of axonal regrowth below the level of injury as well as an inability to reinitiate spinal neurogenesis. However, some anamniotes including the zebrafish *Danio rerio* exhibit both sensory and functional recovery even after complete transection of the spinal cord. The adult zebrafish is an established model organism for studying regeneration following spinal cord injury, with sensory and motor recovery by 6 weeks post-injury. To take advantage of *in vivo* analysis of the regenerative process available in the transparent larval zebrafish as well as genetic tools not accessible in the adult, we use the larval zebrafish to study regeneration after spinal cord transection. Here we demonstrate a method for reproducibly and verifiably transecting the larval spinal cord. After transection, our data shows sensory recovery beginning at 2 days post-injury (dpi), with the C-bend movement detectable by 3 dpi and resumption of free swimming by 5 dpi. Thus we propose the larval zebrafish as a companion tool to the adult zebrafish for the study of recovery after spinal cord injury.

Video Link

The video component of this article can be found at http://www.jove.com/video/51479/

Introduction

Major trauma to the human spinal cord often results in permanent paralysis and loss of sensation below the level of injury, due to the inability to regrow axons or reinitiate neurogenesis[1,2]. In contrast to mammals, however, anamniotes including salamanders and zebrafish (*Danio rerio*) show robust recovery even after complete spinal cord transection[3,4].

The adult zebrafish is a well-established model for studying the recovery process following spinal cord injury[5-7]. Following complete spinal cord transection, reestablishment of sensory and locomotive function is observed in the adult zebrafish by 6 weeks post-injury[8]. In order to examine the regenerative process *in vivo*, we turned to the transparent larval zebrafish[9].

Here we present a method to transect the spinal cord of a 5 days post-fertilization (dpf) larval zebrafish using a beveled microinjection pipette as a scalpel, modified from Bhatt, *et al.*[10] This method supports high throughput, low mortality, and reproducibility. With practice, 300 larvae/hr can be transected, and over 6 months of transections, including over 3,600 animals, 98.75% ± 0.72% survived until 7 days post-injury (dpi). Our data shows rapid recovery of sensory and locomotion as well: at 1 dpi, all movement by the injured fish is driven by pectoral fin locomotion only. However, larvae begin to respond to tungsten needle touch caudal to transection by 2 dpi, reestablish C-bend movement by 3 dpi, and display predatory swimming by 5 dpi[11]. Using antibody staining against acetylated tubulin, we have confirmed that axons are absent from the injury site at 1 dpi, but have crossed the injury site by 5 dpi. We believe this protocol will provide a valuable technique for the study of axonal regrowth and neurogenesis in the spinal cord following injury.

Protocol

Zebrafish were raised and bred according to standard procedures; experiments were approved by the University of Utah Institutional Animal Care and Use Committee.

1. Preparation of Surgery Plates

1. Make surgery plates using 60 mm Petri dishes and Sylgard 184 Silicone Elastomer Kit, following manufacturer's instructions. Fill dishes no more than half-full and allow to polymerize. Store covered at room temperature.

2. Preparation of Micropipettes

1. Fabricate micropipettes by heating and pulling thin-wall borosilicate capillary tubing in a micropipette puller using the same settings for making microinjection needles.

2. Under a dissection microscope, snap off tip of micropipette to approximately 200 µm in diameter with forceps.
3. Bevel broken edge with a microgrinder initially to 35°, followed by a second beveling at 25°. Ensure tip is sharp and smooth. Store finished beveled micropipette in a Petri dish on a small amount of clay.

3. Preparation of Zebrafish Larvae

1. 7 days prior to surgery, set up mating tanks of male and female zebrafish.
2. Collect embryos the following morning, 3 hr after the lights come on to ensure maximum yield. If using a transgenic reporter line such as *Tg(elevl3:eGFP)*^{knu3}, sort fertilized embryos 100/100 mm plate in 25 ml of E3 at 28.5 °C. If using wildtype, sort fertilized embryos 25/100 mm plate in 25 ml of E3 at 28.5 °C.
3. If using a reporter line, screen embryos for fluorescent expression at 48 hpf. Allow identified embryos to mature at a density of 25/100 mm plate in 25 ml of E3 at 28.5 °C.
4. When larvae are 5 dpf, prepare surgery plate by covering Sylgard with E2 + 10 mg/L Gentamycin Sulfate (GS) + Tricaine.
    1. Prepare recovery dish by adding 25 ml E2 + GS to a 100 mm Petri dish.
    2. Prepare scalpel by taping together three swabs. This will form a triangular tool with three grooves.
    3. Mount a prepared micropipette on the swabs by taping it into one of the grooves.
5. If reusing micropipettes, flush until clear with E2 + GS using a 1ml syringe and a 27 G needle prior to mounting on swabs.

4. Surgery

1. Anesthetize 1 plate of larvae at a time (25 fish) with Tricaine. Fish are sufficiently anesthetized when they no longer exhibit touch response. It is important that fish are completely anesthetized prior to surgery, otherwise they will twitch when the scalpel touches them. Surgery is performed under a dissection microscope.
2. Transfer larvae to surgery plate.
    1. Under maximum magnification, rotate one larva at a time so that it lies on its side with its back closest to the hand holding the scalpel.
    2. Position forceps so that they rest on the Sylgard, angled over the width of the larva.
    3. Bracing the glass scalpel against one of the arms of the forceps, cut into the dorsal lateral face of the larva at the level of the anal pore, being sure not to cut beyond the ventral edge of the notochord. Twist the scalpel to sever the spinal cord.
    4. Repeat with remaining larvae.
    Note: if a larva bleeds, it will not recover from the surgery. Immediately remove the larva from the surgery plate and euthanize it via Tricaine overdose.
3. Once surgery on the batch of larvae is complete, transfer injured animals to the recovery plate. This is to support the clearing of anesthesia.
    1. Caution: when collecting injured larvae for transfer, make sure they are collected head or tail first: do not stress the injury site by bending the larvae.
    Note: All devices used for surgery can be reused, including the micropipettes.

5. Recovery

1. Transfer injured larvae from the recovery plate to 100 mm plates filled with 25 ml E2 + GS at a density of 25/plate. Allow to recover in a 28.5 °C incubator.
2. Check plates daily, removing sick and dead animals. Do not change the media until *Coleps* (freshwater protozoa) are visible in the media. When changing the media, do not transfer the fish to a new plate; instead, remove as much media as possible and flood the same plate with new media. Repeat as necessary to reduce *Coleps* population.
3. Feed daily with a small amount of powdered fry food.
Note: Live food (*e.g.*, paramecia or rotifers) cannot be fed to injured larvae until after they have recovered locomotion. Otherwise, the live food will colonize the injury site and kill the larvae.

Representative Results

To reduce severity of tissue damage surrounding the injury site, proper beveling of the micropipette is critical. **Figure 1A** shows a correctly beveled tip. Using a tip that is too wide (**Figure 1B**) tends to result in higher fatalities due to the increased likelihood of nicking the dorsal aorta, while a tip that is too narrow (**Figure 1C**) tends to glance off the skin rather than cutting tissue.

To practice this technique, it is advantageous to use a reporter line such as *Tg(elevl3:eGFP)*^{knu3} to visualize the spinal cord. **Figure 2A** shows a completely transected spinal cord of a live *Tg(elevl3:eGFP)* zebrafish at 1 dpi, while **Figure 2B** shows the same live fish at 3dpi. **Figures 2C** and **2D** show higher magnifications of the injury site at 3 dpi in fixed *Tg(dbx1a:eGFP)* fish having complete (**Figure 2C**) or incomplete (**Figure 2D**) spinal cord transection. Note the contiguous region of neuron labeling along the ventral edge of the spinal cord (yellow arrow).

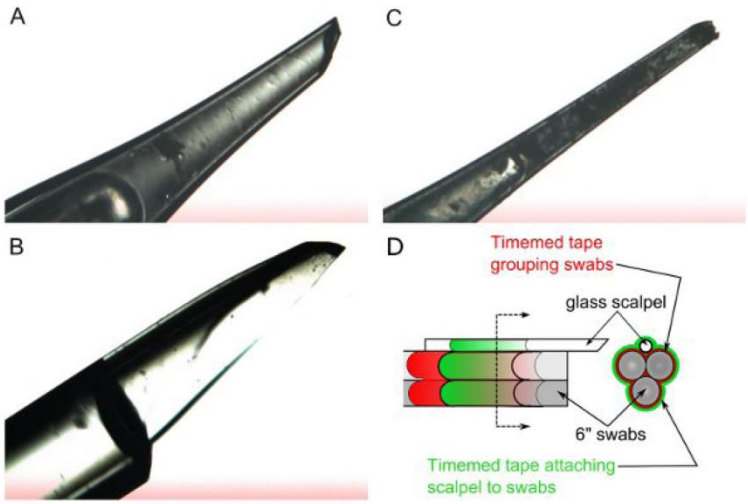

Figure 1. Comparison of scalpel edges. A shows a correctly beveled micropipette tip suitable for surgery. This size is readily cleaned for reuse. **B** shows a beveled micropipette tip too wide for surgery on a 5 dpf larva. **C** is an example of a tip that is too narrow. This size is very difficult to clean for reuse, and tends to promote a sawing action of transection instead of cutting. **D**: cartoon of the lesioning tool assembly. Three 6" swabs are nested into a pyramidal shape and taped together. The scalpel rests in one of the grooves formed by the three swabs, and is taped in place.

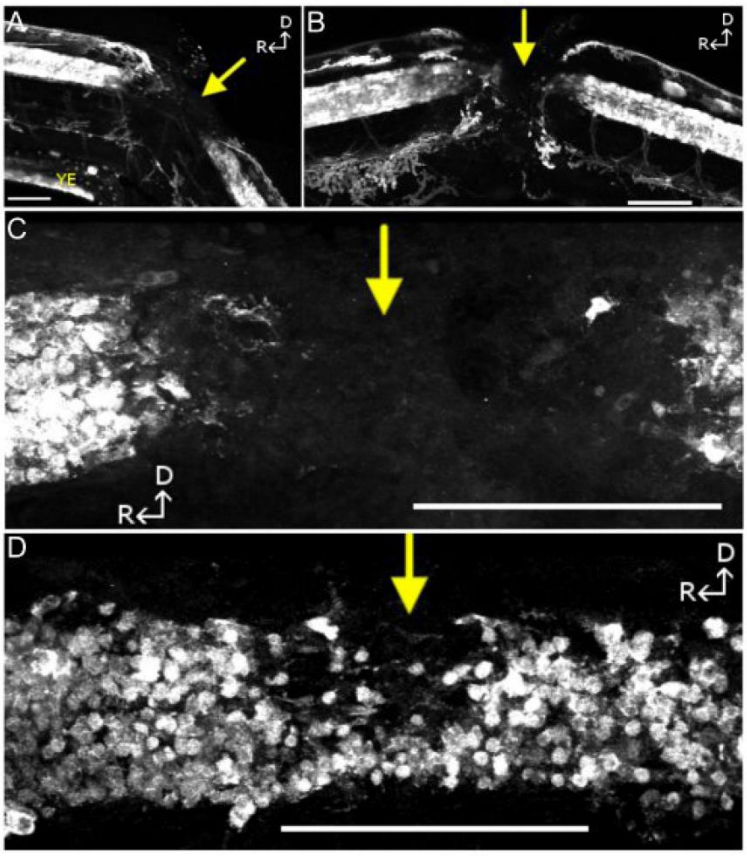

Figure 2. Verifying complete transection. Fluorescent confocal microscopy was used to image live *Tg(elavl3:eGFP)* fish *in vivo* at 1 dpi (**A**) and 3dpi (**B**). To confirm complete transection, these image stacks were then processed in ImageJ (rsbweb.nih.gov) to generate Maximum Intensity Projections (MaxZ) as shown in **A**-**B**. **C**-**D** show MaxZ projections of HuC/D labeled *Tg(dbx1a:eGFP)* fish at 3 dpi with complete spinal transection (**C**) or incomplete transection (**D**). Yellow arrows identify injury site, D=dorsal, R=rostral. Scale bar = 100 µm.

Discussion

When initially learning this technique, we recommend attempting no more than 50-100 transections in a single session. After mastering this technique, we are able to transect up to 300 embryos per hr; however, this level of throughput requires a few months of weekly practice. We also recommend practicing with a reporter line and verifying complete transection until the incidence of incomplete spinal cord transection is reduced to less than 1%.

Spinal cord transection in the adult zebrafish is a well-established and robust technique for studying axonal regrowth and neurogenesis after injury. By moving this analysis into the larval organism, we are able to examine recovery *in vivo*. Additionally, we are also able to utilize genetic tools not available in the adult zebrafish to examine the roles of various genes in the regenerative process, *e.g.*, *Tcf7l1a*[12].

Originally developed to study neurogenesis following spinal cord transection, this technique can also be used to examine recovery of sensory function: injured animals show a response to touch caudal to the injury site by 2 dpi, and axons have crossed the injury site by 5 dpi.

Disclosures

The authors have nothing to disclose.

Acknowledgements

We are indebted to the University of Utah zebrafish facility for animal husbandry. R.I.D. was supported by NIH R56NS053897, and L.K.B. was a predoctoral trainee supported by the HHMI Med-Into-Grad initiative.

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CHAPTER 3

RADIAL GLIAL PROGENITORS REPAIR THE ZEBRAFISH SPINAL CORD FOLLOWING TRANSECTION

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Radial glial progenitors repair the zebrafish spinal cord following transection

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ABSTRACT

In mammals, spinal cord injury results in permanent sensory–motor loss due in part to a failure in reinitiating local neurogenesis. However, zebrafish show robust neuronal regeneration and functional recovery even after complete spinal cord transection. Postembryonic neurogenesis is dependent upon resident multipotent progenitors, which have been identified in multiple vertebrates. One candidate cell population for injury repair expresses *Dbx1*, which has been shown to label multipotent progenitors in mammals. In this study, we use specific markers to show that cells expressing a *dbx1a:GFP* reporter in the zebrafish spinal cord are radial glial progenitors that continue to generate neurons after embryogenesis. We also use a novel larval spinal cord transection assay to show that *dbx1a:GFP*$^+$ cells exhibit a proliferative and neurogenic response to injury, and contribute newly-born neurons to the regenerative blastema. Together, our data indicate that *dbx1a:GFP*$^+$ radial glia may be stem cells for the regeneration of interneurons following spinal cord injury in zebrafish.

Introduction

During vertebrate embryogenesis, neurons of the central nervous system (CNS) are initially derived from neuroepithelial progenitors, some of which transform into radial glia (Mori et al., 2005). By the end of embryogenesis most mammalian radial glia differentiate as astrocytes (Rakic, 2003). However in anamniotes radial glia persist widely in the CNS (García-Verdugo et al., 2002; Naujoks-Manteuffel and Roth, 1989; Zupanc and Clint, 2003), and their continued presence has been implicated in the striking ability of these animals to regenerate following injury (Chernoff et al., 2003; Hui et al., 2010; Rehermann et al., 2011). Thus radial glia have been suggested to represent an endogenous neural stem cell population.

In contrast to the permanent loss of sensory and motor function after spinal cord injury observed in mammals, urodele amphibians and teleost fish regenerate lost tissue and reestablish damaged connections, restoring function to nearly pre-injury levels (Chernoff et al., 2002; Kuscha et al., 2012). In addition to axonal regrowth after spinal cord transection in adult zebrafish (Becker et al., 1997, 2004; Goldshmit et al., 2012; Schweitzer et al., 2007), spinal lesion triggers generation of motoneurons and interneurons, with pre-injury levels restored by 6–8 weeks post injury (wpi, Reimer et al., 2008). While *olig2*$^+$ radial glia represent a pool of motoneuron progenitors that contribute to neurogenesis after lesion, the identity and behavior of other progenitor populations remain unknown.

In vertebrates, *Dbx* genes encode a family of homeodomain transcription factors expressed in the intermediate spinal cord that is necessary for spinal cord development (Jessell, 2000; Lu et al., 1992). *Dbx1*-expressing cells predominately produce Evx1/2$^+$ interneurons (Pierani et al., 2001), but also generate radial glia, astrocytes and oligodendrocytes (Fogarty et al., 2005). In mouse, *Dbx1* expression is not detectable beyond E16.5, suggesting that mammalian *Dbx1*$^+$ progenitors terminally differentiate (Fogarty et al., 2005). Zebrafish have two *Dbx1* orthologs, *dbx1a* and *dbx1b*, which are similarly expressed in the intermediate spinal cord (Gribble et al., 2007; Seo et al., 1999); however, their lineage is uncharacterized. Based on the multipotency of *Dbx1*$^+$ progenitors in amniotes, and the persistence of radial glia in zebrafish, we hypothesized that *Dbx1*-expressing cells might represent a population that could contribute to regeneration of the spinal cord following injury.

We previously generated a *dbx1a:GFP* transgenic reporter line (Gribble et al., 2009), and showed that GFP expression colocalized with endogenous *dbx1a* expression in embryonic spinal progenitors. In this study, we characterize the identity of *dbx1a:GFP* expressing cells and their progeny in the embryonic and larval zebrafish, and their response to spinal cord transection. We show that *dbx1a* mRNA expression persists beyond embryogenesis, and that the *dbx1a:GFP* reporter transgene labels a neurogenic spinal progenitor population. We also show that *dbx1a:GFP* expressing cells are slowly dividing neural progenitors that increase their rate of neurogenesis beyond basal levels in response to transection. Together, our data suggest that *dbx1a:GFP*$^+$

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0014-4886/

radial glia may represent a neural stem cell population in the postembryonic spinal cord that can be activated in response to injury.

Materials and methods

Fish strains and staging

Embryos were obtained from wildtype (AB*), *Tg(olig2:dsRed)*^{vu19}, *Tg(elavl3:EGFP)*^{knu3}, and *Tg(-3.5 dbx1a:EGFP)*^{zf3} crosses (Gribble et al., 2009; Kucenas et al., 2008; Park et al., 2000, 2007), and staged according to Kimmel et al. (1995). Zebrafish were raised and bred according to standard procedures; experiments were approved by the University of Utah Institutional Animal Care and Use Committee.

In situ hybridization

Embryos were fixed in fresh 4% paraformaldehyde (PFA) overnight at room temperature, washed in PBS, decapitated and coarsely chopped. *In situ* hybridization was performed as described previously (Oxtoby and Jowett, 1993). For sectioning, embryos were cryoprotected in sucrose, embedded in OCT, and sectioned at 20 μm thickness on a Leica CM3050 cryostat. Images were taken on an Olympus BX51WI compound microscope using an Olympus Microfire camera. Images were processed using the GNU Image Manipulation Program (GIMP.org).

Immunohistochemistry

Embryos up to 48hpf (hours post fertilization) were fixed in fresh 4% PFA for 3 h at room temperature then overnight at 4 °C; embryos over 48hpf were fixed for 1 h at room temperature. After fixation, embryos were washed in PBS, cryoprotected in sucrose, embedded in OCT and sectioned at 12 μm or 50 μm thickness on a Leica CM3050 cryostat. For BrdU antigen retrieval, thick sections were incubated at room temperature for 90 min in 2 N HCl. For PCNA antigen retrieval, thin sections were incubated in 100 °C 10 mM sodium citrate buffer, pH 6.0. Primary antibodies used were: rabbit anti-GFP (1:5000, Invitrogen #A-11122), chicken anti-GFP (1:1000, Aves #GFP-1020), mouse anti-HuC/D (1:500, Invitrogen #A-21271), chicken anti-BrdU (1:500, ICL #CBDU-65A-Z), mouse anti-PCNA (1:1000, Sigma #p8825), rabbit anti-PCNA (1:100, Santa Cruz Biotechnology #F2212), rabbit anti-Sox3 (1:200, a gift from Dr. Mike Klymkowsky, University of Colorado-Boulder), rabbit anti-Sox3 (1:200, Pierce Custom Antibodies and Peptides), rabbit anti-DsRed (1:200, Clontech #632496), and mouse zrf-1 (1:200, Zebrafish International Resource Center #zrf-1). Secondary antibodies used were: goat anti-rabbit 488 (1:200, Invitrogen #A-11008), goat anti-rabbit 568 (1:200, Invitrogen #A-11041), goat anti-rabbit cy3 (1:200, Jackson ImmunoResearch #111-165-003), goat anti-mouse 633 (1:200, Invitrogen #A-21050), goat anti-mouse cy3 (1:200, Jackson ImmunoResearch #115-165-003), goat anti-chicken 488 (1:200, Invitrogen #A-11039), goat anti-chicken 633 (1:200, Invitrogen #A-21103), and donkey anti-chicken 488 (1:200, Jackson ImmunoResearch #703-485-155). Hoechst 33342 was added to secondary antibodies to visualize nuclei.

Confocal microscopy

Sections were imaged using an Olympus FV-1000XY confocal microscope using a 60x oil-immersion objective. Images were processed using ImageJ (http://rsbweb.nih.gov/ij) and GIMP (gimp.org). Projections were generated using FluoRender (Wan et al., 2012).

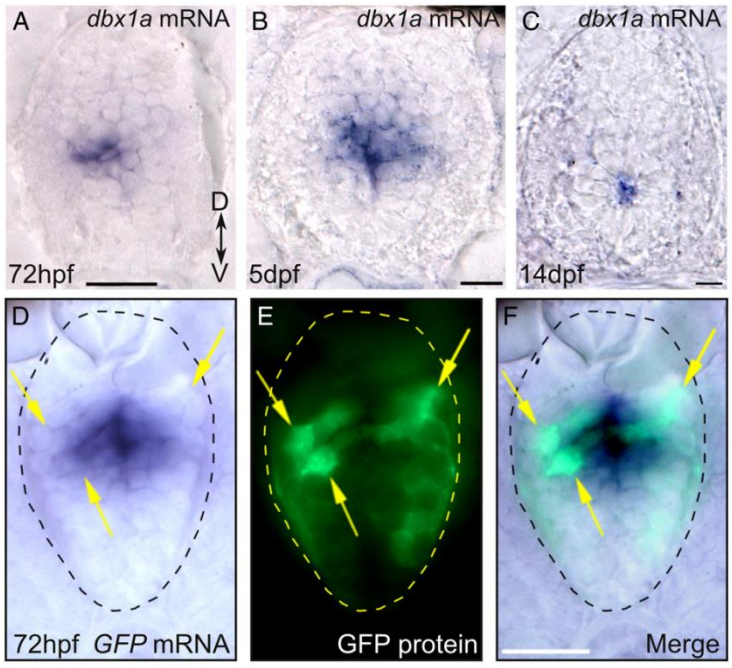


Fig. 1. Expression of *dbx1a* mRNA and *dbx1a:GFP* reporter. (A–C) *In situ* hybridization shows that *dbx1a* mRNA is expressed in the intermediate spinal cord through 14dpf. (D–F) In 3dpf *dbx1a:GFP* embryos, GFP protein expression is observed in lateral cells negative for *GFP* mRNA (arrows), suggesting that perdurance of protein can be used to trace the lineage of reporter-expressing cells. Scalebars = 10 μm.

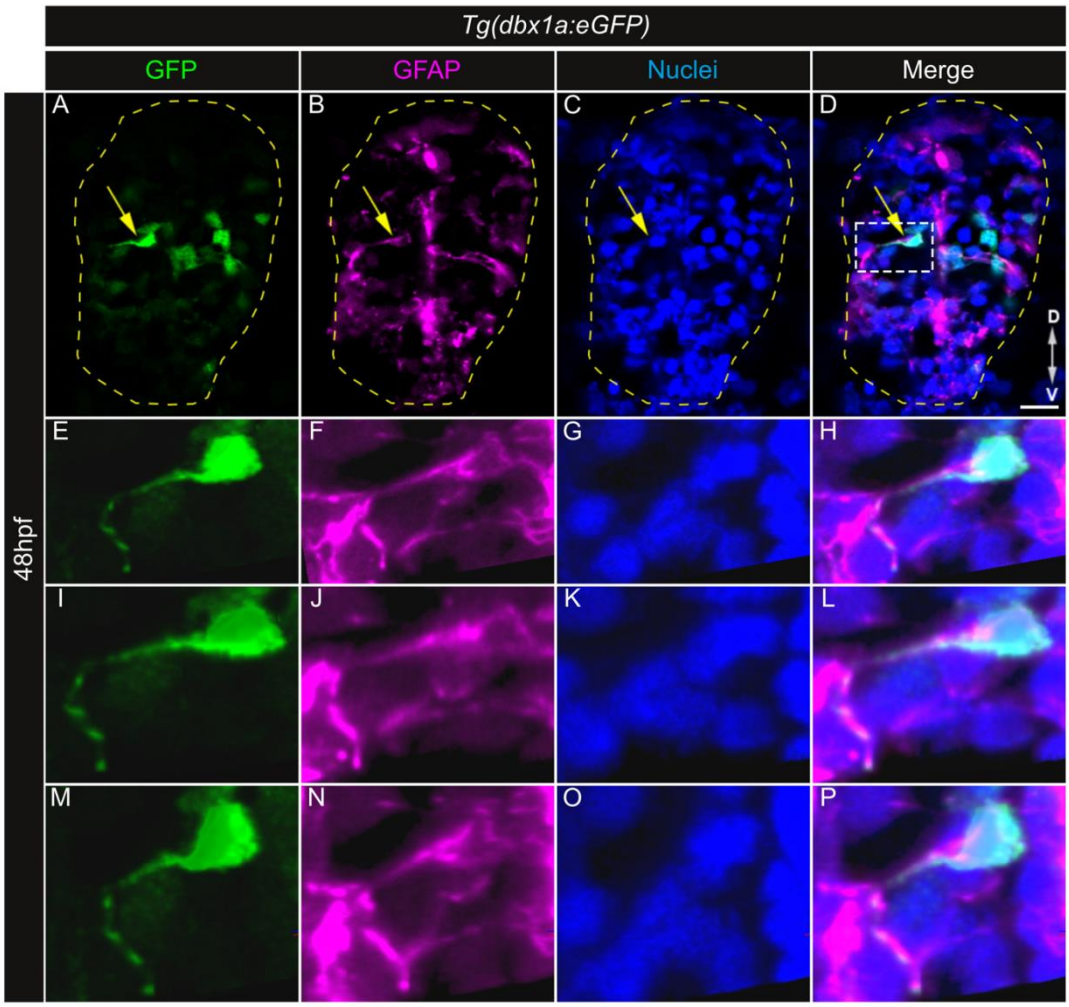


Fig. 2. GFP and GFAP colocalization. (A–D) Colocalization of *dbx1a:GFP* with GFAP at 48hpf in a single confocal slice. Arrow denotes a double-labeled cell based on colocalization of GFP with nuclear staining and cortical GFAP around the nucleus, and colocalization of GFP and GFAP in the process. White dashed box in (D) denotes region of interest in (E–P). (E–H) Maximum Z-projection showing colocalization of GFP and GFAP. (I–P) Rotated projection views showing colocalization of GFP and GFAP. Scale bar = 10 μm.

Spinal cord transection

Tg(elavl3:EGFP)^{knu3} or *Tg(-3.5dbx1a:EGFP)*^{zd3} 4dpf (days post fertilization) embryos were raised in E2 medium + 0.2 mM phenothiourea (Sigma) + 10 mg/L gentamycin sulfate (Amresco), treated with 10 mM BrdU (Sigma) for 24 h then immediately lesioned at 5dpf as described previously (Bhatt et al., 2004; Briona and Dorsky, 2013) and transferred back into E2-PTU-gentamycin sulfate media. Briefly, microinjection glass pipettes were broken, beveled and used as a scalpel. Fish were anesthetized with 0.016% Tricaine (Sigma), braced with microforceps, and lesioned by driving a glass scalpel through the spinal cord at the level of the anal pore, and moved dorsally to sever the entire spinal cord. Sham treated animals were anesthetized and braced as described, and touched with the glass scalpel on the dorsal flank at the level of the anal pore without breaking the skin.

Quantification of dbx1a^{+} progeny

Five non-consecutive transverse 12 μm cryosections from five *Tg(-3.5dbx1a:EGFP)*^{zd3} embryos were quantified for antibody co-localization based on MaxZ projections generated with ImageJ (http://rsbweb.nih.gov/ij). *dbx1a:eGFP*$^{+}$ cells were analyzed based on single-slice verification of colocalization with nuclear staining, verifying that the contiguous area of GFP expression was at least as large as the nucleus (Supplemental Fig. 1).

Quantification of spinal cord regeneration

Nineteen *Tg(elavl3:EGFP)*^{knu3} animals were injured at 5dpf, and imaged every other day until the end of the experiment. For BrdU labeling studies, 50 μm sagittal cryosections from 95 *Tg(-3.5 dbx1a:EGFP)*^{zd3}

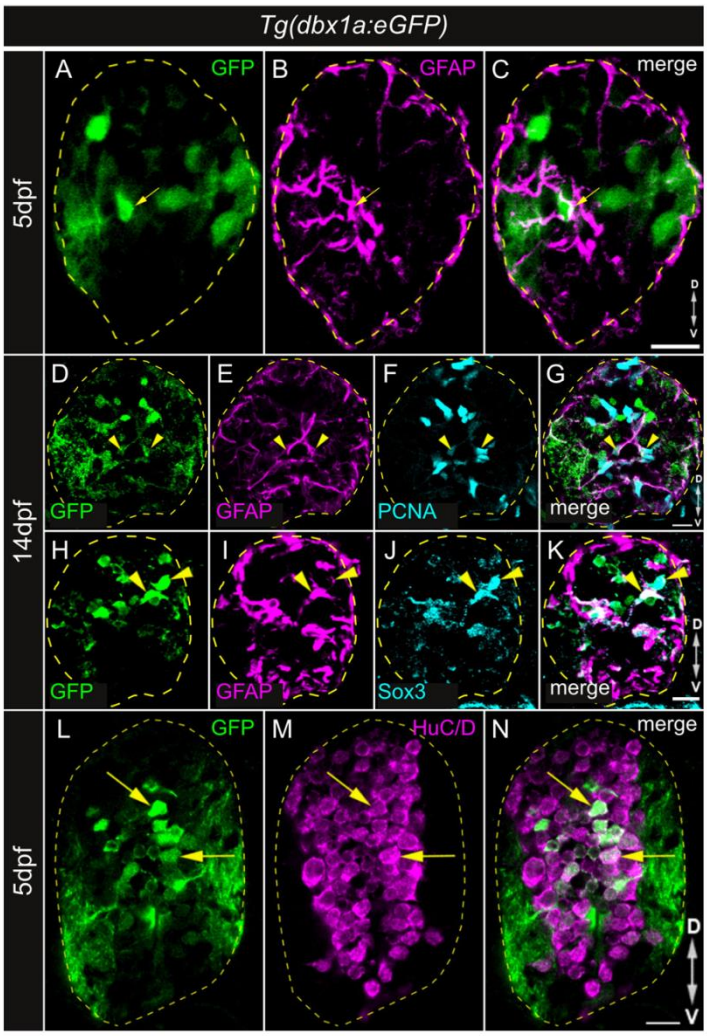


Fig. 3. *dbx1a:GFP*$^+$ progenitors persist beyond embryogenesis. (A–C) *dbx1a:GFP*$^+$ cells contribute to a GFAP$^+$ glial population. Arrow marks a double-labeled cell. (D–G) *dbx1a:GFP*$^+$ glia remain proliferative beyond embryogenesis. Arrowheads mark triple-labeled cells. (H–K) *dbx1a:GFP*$^+$ glia persist as Sox3$^+$ neural progenitors. Arrowheads mark triple-labeled cells. (L–N) *dbx1a:GFP*$^+$ cells contribute to an intermediate HuC/D$^+$ neuronal population. Arrows mark double-labeled cells. Scalebars = 10 μm; all images are single optical sections.

embryos were quantified for antibody colocalization at 1-9dpi (days post injury). MaxZ projections were stitched together using ImageJ and GIMP. The plane of injury was identified, the spinal cord was outlined, and the leading edge of a healthy spinal cord proximal to the injury site was labeled as 0 μm. Three regions in the spinal cord were characterized: a 30 μm region 200 μm rostral to the leading edge of the uninjured spinal cord was identified as "rostral," a 30 μm region immediately adjacent to the rostral stump was identified as "proximal", and the region adjacent to the leading edge of the healthy spinal cord at the injury site was characterized as "blastema." In sham-treated animals, the spinal cord at the level of the anal pore was labeled as 0 μm, and the rostral and proximal regions were identified relative to this point as described previously.

Statistical analysis

All results were expressed as mean ± SEM. Two-tailed two-sample equal variance Student t-tests were calculated using Excel (Microsoft Corporation) or R (GNU S). Differences were considered significant at $p < 0.05$.

Results

dbx1a mRNA and reporter expression persists beyond embryogenesis

Previous research in our laboratory using whole mount *in situ* hybridization showed that *dbx1a* was expressed in the intermediate

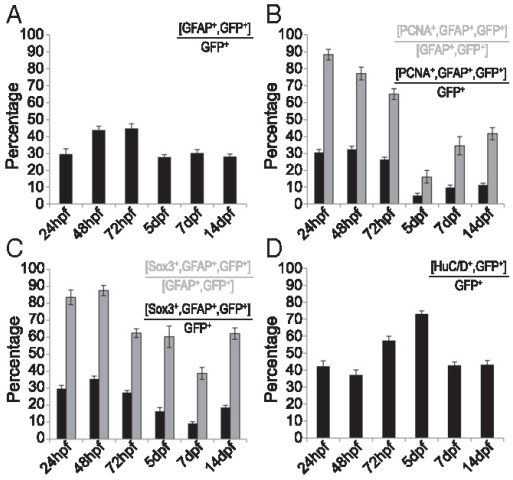


Fig. 4. A stable population of *dbx1a:GFP*$^+$ progenitors persists in the spinal cord. (A) Percentage of *dbx1a:GFP*$^+$ progenitors that are GFAP$^+$. (B) Percentage of PCNA$^+$ cells in the overall *dbx1a:GFP*$^+$ population (black), and among the GFAP$^+$ subpopulation (gray). (C) Percentage of Sox3$^+$ neural progenitors in the overall *dbx1a:GFP*$^+$ population (black), and among the GFAP$^+$ subpopulation (gray). (D) Percentage of HuC/D$^+$ neurons in the *dbx1a:GFP*$^+$ population. n = 25 at each timepoint; error bars = SEM. Scalebar = 10 μm.

spinal cord by 15 h post fertilization (hpf), but was no longer detectable by 72hpf (Gribble et al., 2007). However, probe penetration becomes difficult beyond 48hpf due to mesenchymal condensation around the spinal cord (Bader et al., 2009; Simmons and Appel, 2012; Thisse et al., 2004) and cartilage formation at later stages (Sisson and Topczewski, 2009; Williams et al., 2000). Using coarsely chopped wildtype trunk sections we re-examined *dbx1a* mRNA expression from 3 days post fertilization (dpf) through 14dpf, and at all timepoints we observed expression in the intermediate spinal cord (Figs. 1A–C). These data suggested that *dbx1a*-expressing cells may have a role beyond embryonic neurogenesis.

To determine the identity and ultimate fate of *dbx1a*-expressing cells, we used the *Tg(-3.5dbx1a:EGFP)*^{zd3} transgenic line previously characterized in our laboratory (Gribble et al., 2009). In order to test whether GFP protein perdurance in this line could be used as a marker for lineage tracing experiments, we examined the expression of *GFP* mRNA and GFP protein in 3dpf spinal sections. We found that mRNA expression was strongest in cells of the intermediate spinal cord immediately adjacent to the central canal and weak or absent in the lateral spinal cord where postmitotic motor neurons reside (Fig. 1D). In the same sections, we observed GFP protein overlapping with mRNA expression, but also more laterally in cells negative for *GFP* mRNA (Figs. 1E, F). Thus, we conclude that expression of GFP protein in postmitotic neurons is due to perdurance of the protein after cessation of mRNA expression, and that GFP protein expression marks both *dbx1a*-expressing progenitors and their immediate progeny.

dbx1a:GFP$^+$ *radial glial progenitors are neurogenic beyond embryogenesis*

To determine which cell types express *dbx1a:GFP* during embryonic and larval stages, we examined the coexpression of GFP with cell type specific markers in transgenic fish (Supplemental Fig. 1). Zebrafish embryogenesis is considered complete by 5dpf, as this marks the developmental timepoint when feeding begins (Westerfield, 2000). At 24hpf, 30% of GFP$^+$ cells were co-labeled by the zrf-1 antibody that recognizes GFAP, a marker of radial glia (Trevarrow et al., 1990). Because GFAP is a cytoskeletal intermediate filament while GFP is nuclear and cytoplasmic, the two antigens do not exhibit an identical cellular expression (Barresi et al., 2010). To confirm colocalization of GFP and GFAP, individual cells were examined for a cortical ring of GFAP expression around a GFP$^+$ nucleus, as well as axonal extensions that were both GFAP$^+$ and GFP$^+$ (Fig. 2). The peak colocalization of GFP and GFAP occurred at 48hpf and was maintained at 44% until 72hpf. At 5dpf, levels of GFP and GFAP coexpression returned to 30%, and remained steady until 14dpf (Figs. 3A–C; Fig. 4A). To further characterize this population, we examined colocalization of GFAP, GFP, and PCNA to identify proliferating radial glia, and GFAP, GFP, and Sox3 to identify neural progenitors (Goldman, 2003; Kim and Dorsky, 2011; Malatesta et al., 2000; Wang

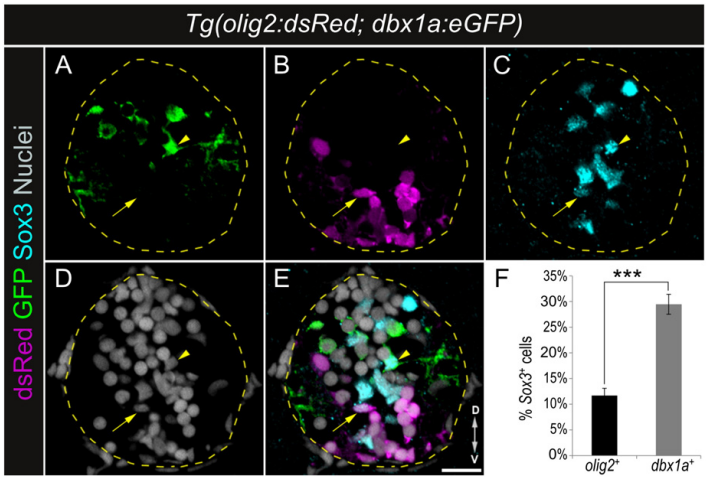
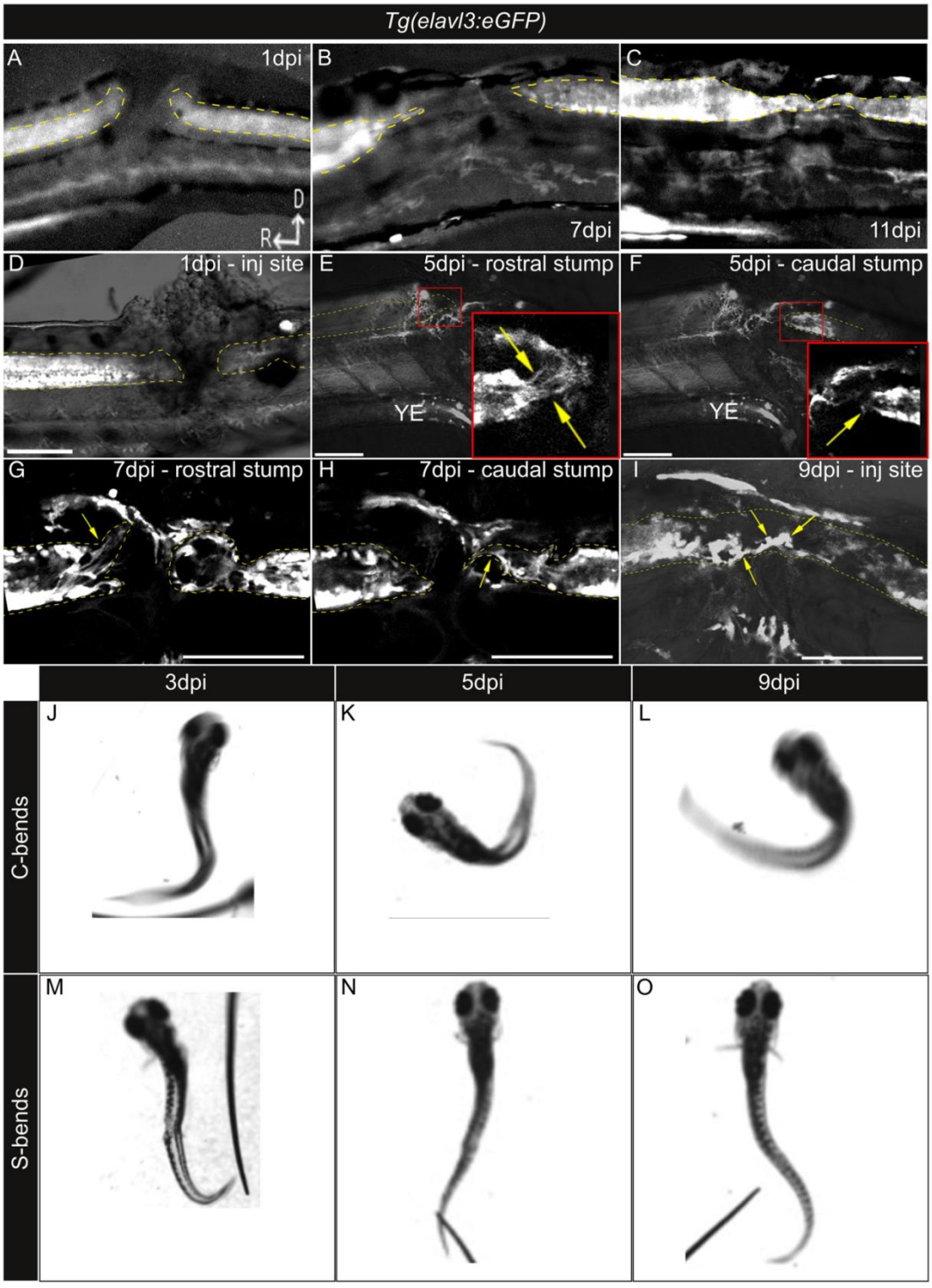


Fig. 5. *dbx1a:GFP*$^+$ and *olig2:dsRed*$^+$ mark two independent progenitor populations. (A–E) At 5dpf, the *dbx1a:GFP*$^+$ and *olig2:dsRed*$^+$ populations do not overlap, suggesting they constitute separate lineages. Both populations contain Sox3$^+$ neural progenitors (arrowhead and arrow). (F) At 5dpf, more *dbx1a:GFP*$^+$ cells than *olig2:dsRed*$^+$ cells express Sox3. N = 25; error bars = SEM, scalebar = 10 μm.


Tg(elavl3:eGFP)
A
1dpi
D
R
B
7dpi
C
11dpi
D
1dpi - inj site
E
5dpi - rostral stump
YE
F
5dpi - caudal stump
YE
G
7dpi - rostral stump
H
7dpi - caudal stump
I
9dpi - inj site
3dpi
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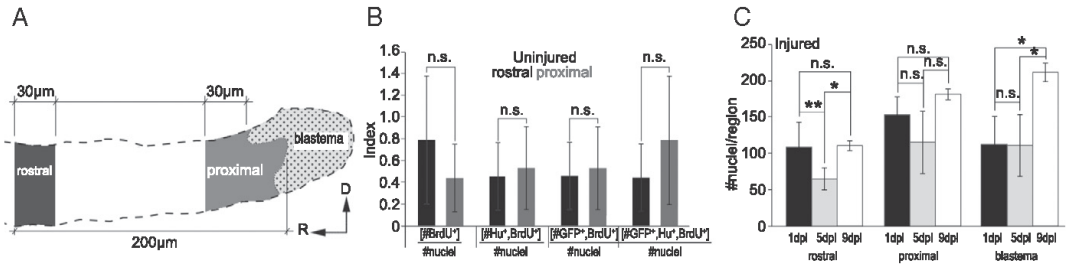


Fig. 7. Definition of responsive zones following transection. (A) Schematic of zone identification in transected rostral spinal cord. The blastema was defined as the area containing non-contiguous HuC/D$^+$ neurons. Rostral and proximal zones were defined by their position relative to the blastema. (B) Rostral (black) and proximal (gray) zones in the uninjured spinal cord are similar in proliferation and rate of neurogenesis, suggesting that the larval spinal cord primarily exhibits homeostatic maintenance beyond 5dpf. (C) The number of nuclei in rostral and proximal zones remains relatively unchanged following transection; however, there is a significant increase in the number of nuclei in the blastema by 9dpi. n = 5–7 fish per condition, per timepoint. Error bars = SEM; *p < 0.05, **p < 0.01, ***p < 0.001.

et al., 2006). At 24hpf, 90% of GFP$^+$ radial glia were also PCNA positive. This percentage dropped over time to 18% at 5dpf, after which the percentage of GFP$^+$ radial glia that were also PCNA$^+$ returned to 40% at both 7dpf and 14dpf (Figs. 3D–G'; Fig. 4B). We also found several GFP$^+$ and GFAP$^+$ radial glia positive for Sox3 expression at the central canal starting at 24hpf, and at all timepoints examined (Figs. 3H–K'; Fig. 4C). Together, these data suggest that *dbx1a:GFP*$^+$ cells persist as proliferating neural progenitors beyond embryogenesis.

We next asked whether *dbx1a:GFP*-expressing progenitors contribute to the neuronal population by examining HuC/D coexpression with GFP (Figs. 3L–N). At 24hpf, 42% of the GFP$^+$ cells were also HuC/D$^+$ (Fig. 4D). This coexpression expanded by 5dpf to a maximum of 73% of GFP$^+$ cells, and returned to baseline levels at 7dpf, with 43% of GFP$^+$ cells also HuC/D$^+$. Consistent with our observations that GFP protein expression perdures in the progeny of *dbx1a:gfp*$^+$ radial glia, we found that a 2-hour pulse of BrdU did not immediately label any GFP$^+$ neurons, while an additional 24-hour chase allowed us to label these cells (data not shown). Taken together, these data show that *dbx1a:GFP*$^+$ progenitors continue to divide and produce neurons beyond the end of embryogenesis, suggesting that they represent a potential source of regenerative potential following injury.

dbx1a:GFP$^+$ *progenitors are distinct from the olig2*$^+$ *population*

Progenitors in the ventral spinal cord expressing *olig2* have been proposed as neural stem cells in post-embryonic zebrafish (Park et al., 2007; Reimer et al., 2008). To confirm that *dbx1a:GFP* expressing cells are separate from the *olig2*$^+$ population, we crossed *Tg(olig2:dsRed)*^{vu19} and *Tg(-3.5 dbx1a:EGFP)*^{zd3} fish and examined transgene colocalization in offspring. At earlier timepoints we did not observe any dsRed, GFP double positive cells (data not shown), and even at 5dpf, the peak of neurogenesis for *dbx1a:GFP*$^+$ cells, dsRed did not colocalize with GFP (Figs. 5D–F), suggesting that *dbx1a:GFP*$^+$ cells are not a subset of *olig2:dsRed*$^+$ progenitors. To determine the relative contributions of both lineages to neural progenitors in the developing spinal cord, we labeled 5dpf *olig2:dsRed;dbx1a:GFP* larvae with the neural progenitor marker Sox3. Colabeling showed that at 5dpf, 29.46% ± 1.93 of Sox3$^+$ cells were GFP$^+$ while 11.69% ± 1.41 of Sox3$^+$ cells were dsRed$^+$. These data suggest that while both populations contribute to the neural progenitor population, *dbx1a:GFP*$^+$ cells represent a comparatively larger progenitor source.

Larval zebrafish rapidly regenerate their spinal cord following transection

We next wanted to determine whether *dbx1a:GFP* progenitors in the post-embryonic spinal cord were able to regenerate lost neurons post-injury. While the adult zebrafish is a well-established model for studying spinal cord regeneration following transection, crush injury, or laser ablation of cells (Becker et al., 2004; Goldshmit et al., 2012; Hui et al., 2010), we chose to study spinal cord regeneration in larval zebrafish, which are transparent and thus allow an *in vivo* analysis of the regenerative process (Hale et al., 2001). The larval stage begins at 5dpf, which coincides with a decrease in spinal progenitor proliferation and differentiation to a level that remains stable for weeks (Fig. 4 and Park et al., 2007). Therefore, neurogenesis after injury in larvae beyond this timepoint should require reactivation of progenitors into a regenerative program. While anatomical and functional recovery following spinal cord transection in the adult zebrafish takes 6–8 weeks (Reimer et al., 2008), we hypothesized that regeneration after transection would occur faster in the larva, based on maturity and size (Navarro et al., 1988; Sun et al., 2005).

Using a broken beveled micropipette as a glass scalpel, we completely transected the spinal cord of anesthetized 5dpf larvae at the level of the anal pore. Complete transection was verified visually by confirming a complete gap between rostral and caudal cord stumps (Fig. 6A), and physiologically by lack of touch response caudal to the injury site. Sham animals were treated the same as experimental, except that they were only touched with the glass scalpel; no incision was made. Over 95% of transected fish survived the surgery until 7 days post injury (7dpi), at which point about 50% of the survivors died, likely due to the inability to feed as a result of failure to inflate the swimbladder (Haffter et al., 1996). Over 95% of sham treated animals survived until the end of the experiment.

Using *elavl3:GFP* larvae to visualize postmitotic neurons of the spinal cord, we first examined regeneration in live fish using compound fluorescence microscopy (Figs. 6A–C). At 1dpi (6dpf) no regeneration was

Fig. 6. Larval zebrafish recover rapidly from spinal cord transection. (A–C) Compound microscope images of regeneration from a single live larva, using *elavl3:EGFP* to label postmitotic neurons. The spinal cord is marked by a dashed line. (A) At 1dpi there is a clear gap between rostral and caudal stump ends after transection. (B) Processes projecting from the rostral stump into injury site are visible by 7dpi. (C) By 11dpi, the rostral and caudal stumps are contiguous across the injury site. (D–I) Confocal images of regeneration from a single live larva, using *elavl3:EGFP* to label postmitotic neurons. Spinal cord is marked by a dashed line. (D) At 1dpi there is a gap between the rostral and caudal stumps after injury; brightfield overlay shows location of wound site and scar tissue. (E, F) By 5dpi, rostral processes toward the pial surface can be seen (E, inset, arrows), but processes are absent from the caudal end (F, inset, arrow). (G) By 7dpi, numerous processes are visible at the rostral severed end (arrow), while the recovery response of the caudal end (H) resembles the rostral end at 5dpi (arrow). (I) By 9dpi, GFP$^+$ soma are present in the injury site (arrows). All confocal images are single slices except (D), which is a Max-Z projection. Dorsal (D) and rostral (R) are marked in panel (A). Scalebar = 50 μm, YE = yolk extension. (J–O) Functional recovery following spinal cord transections. C-bends and S-bends are inducible at 3dpi (J,M). Magnitude and frequency of movement increases at 5dpi (K, N) and 9dpi (L, O).

visible (Fig. 5A). By 7dpi, neuronal processes from the rostral end of the spinal cord were visible, and appeared to be projecting towards the dorsal pial surface (Fig. 6B). By 11dpi, the severed spinal cord was once again contiguous across the injury site (Fig. 6C). To examine the regeneration process more closely, we visualized injured *elavl3:GFP* larvae *in vivo* using confocal microscopy (Figs. 6D–I). At 1dpi, no regeneration was visible (Fig. 6D). By 5dpi, rostral neuronal processes were visible (Fig. 6E, arrows); however, the regenerative response was delayed on the caudal side of the injury, as no processes were visible at this time (Fig. 6F). At 7dpi, rostral neuronal processes had become more numerous, and caudal processes were visible as well (Figs. 6G, H, arrows). By 9dpi, *elavl3:gfp*$^+$ soma were present in the injury site (Fig. 6F, arrows).

We also examined the recovery of spinal cord function following transection. At 1dpi, all injured fish were nonresponsive to touch on their tail caudal to the injury site with a tungsten needle probe; however, touch rostral to injury site did elicit a startle response (Supplemental Movie 1). By 2dpi, 11/165 fish showed sporadic response to touch caudal to injury site (Supplemental Movie 2). At 3dpi, 28.5% exhibited a sporadic C-bend response to touch caudal to injury site, while 5.1% consistently responded to touch on both sides of the tail caudal to injury site, at all levels tested (Figs. 6J, M; Supplemental Movie 3). Since the C-bend startle response is dependent upon reticulospinal neurons (Burgess and Granato, 2007), we used acetylated tubulin to label spinal axons. At 1dpi, there were no axons crossing the injury site, but by 5dpi, numerous axons crossed the injury gap (Supplemental Fig. 2). By 5dpi, all injured fish still alive exhibited either sporadic (58.31%) or consistent response to touch, with some having resumed voluntary swimming (Figs. 6K, N; Supplemental Movie 4). By 9dpi, all surviving fish showed robust swimming using both pectoral and caudal fins and a consistent startle response to touch caudal to injury site (Figs. 6L, O; Supplemental Movie 5). Together these data show that larval zebrafish are capable of regenerating a transected spinal cord with regenerated axons crossing the injury gap and neuronal soma present at the injury site by 9dpi, reestablishing both sensory and motor function within 9dpi.

dbx1a:GFP$^+$ *progenitors contribute to neurogenesis after spinal cord transection*

To determine whether *dbx1a:GFP*-expressing progenitors undergo a proliferative and neurogenic response following injury, we examined BrdU incorporation and HuC/D expression in *dbx1a:GFP* fish during recovery from spinal cord transection. To avoid potential complications from differences in BrdU accessibility following injury, we chose to label both transected and control larvae *via* incubation in BrdU for 24 h immediately before injury. To facilitate characterization of the recovery process, we defined three zones of examination. The blastema was defined as the active recovery zone closest to the transection with its rostral edge located where neurons became discontinuous (Fig. 7A). The neighboring proximal zone was defined as 30 μm rostral to the blastema, and a rostral zone was defined as 170–200 μm away from the rostral edge of the blastema. We hypothesized that the blastema zone would have the highest rate of sustained neurogenesis after injury, while the proximal zone would have the earliest proliferative response due to a high concentration of reactive progenitors. We also hypothesized that the regenerative response would be local to the injury site, and thus the rostral zone would reflect rates of proliferation and neurogenesis similar to that observed in sham treated animals. Our analysis was limited to the spinal cord rostral to the injury site to focus on the earliest-regenerating cells identified in our initial characterization.

To establish baseline rates of proliferation and neurogenesis in sham-treated animals, 5–8 larvae at 1dpi–9dpi timepoints were examined. No day-to-day differences between proximal and rostral zones (as defined relative to the transection site in injured animals) were observed in comparing the following indices: proliferation (%BrdU$^+$ nuclei), proliferation of *dbx1a:GFP*$^+$ cells (%BrdU$^+$, GFP$^+$ nuclei), rate of neurogenesis (%BrdU$^+$, Hu$^+$ nuclei), and rate of neurogenesis of *dbx1a:GFP*$^+$ cells (%BrdU$^+$, GFP$^+$, Hu$^+$ nuclei) (Fig. 7B). When data were averaged across all nine days and compared, no significant differences between proximal and rostral regions were observed. Thus, both the rates of proliferation and neurogenesis in uninjured animals are stable between 5dpf–14dpf, and could be aggregated together as sham data without regard for position analyzed. To characterize proliferation after injury, we counted the number of nuclei present in each region at 1, 5, and 9dpi (Fig. 7C). In the rostral zone, there was a transient decrease in the number of nuclei at 5dpi. The number of nuclei remained relatively consistent during regeneration in the proximal zone; however, there was a significant increase in the number of nuclei in the blastema by 9dpi.

Representative maximum intensity Z-projections of the neurogenic process are shown in Fig. 8, and colocalization of markers was confirmed using single slices (Supplemental Fig. 3). Many BrdU$^+$ cells were detectable outside of the spinal cord following injury; these are likely to be part of the immune response and dermal and myogenic repair (Suzuki et al., 2005). However, our analysis was limited to the spinal cord. When we examined neurogenesis after injury in *dbx1a:GFP* fish, we observed no newly born neurons or GFP$^+$ cells in the blastema at 1dpi (Figs. 8A–D; A'–D'). At 5dpi GFP$^+$, Hu$^+$ and BrdU$^+$ cells were observed at the proximal zone–blastema transition area (arrows) as well as in the rostral zone (Figs. 8E–H, E'–H'). By 9dpi, multiple neurons were present in the blastema, both GFP$^+$, Hu$^+$, BrdU$^+$ (arrows) and GFP$^+$, Hu$^+$, BrdU$^-$ (arrowheads, Figs. 8I–L, I'–L').

We found that there was an increase in BrdU$^+$ cells in all regions following injury (Figs. 9A–C), and normalizing to the number of nuclei, we were able to conclude that proliferation occurred at all three regions by 5dpi, continuing until 9dpi (Figs. 9D–F). To specifically determine if *dbx1a:GFP*$^+$ cells proliferate in response to injury, we examined them as a subset of total BrdU$^+$ cells (Figs. 9G–I). In the rostral zone, there was a significant increase in the index of BrdU$^+$,GFP$^+$ cells at 1dpi, continuing through 9dpi. In the proximal zone, the index of BrdU$^+$,GFP$^+$ cells showed a trend of increase by 5dpi, with a significant increase at 9dpi. In the blastema, there was a brief reduction in the index of BrdU$^+$,GFP$^+$ cells compared to baseline at 1dpi, which is consistent with the cell death and debris clearing associated with spinal cord transection (Hui et al., 2010). The index of BrdU$^+$,GFP$^+$ cells increased significantly by 5dpi, and remained elevated through 9dpi. These data show that *dbx1a:GFP*$^+$ cells proliferate in response to injury, and that the increase in proliferation is also systemic.

We also found that neurogenesis was increased in response to injury (Figs. 9J–L). In the rostral zone, the number of newly born (BrdU$^+$) neurons in injured fish did not significantly vary from basal levels at all timepoints examined, suggesting that BrdU$^+$ progenitor cells observed in this area do not differentiate in this location. In the proximal zone, a significant increase in the number of neurons born after injury was detected at 5dpi, continuing through 9dpi. Interestingly, a significant increase in the index of HuC/D$^+$,BrdU$^+$ cells was not observed in the blastema until 9dpi, suggesting that the neurons born shortly after injury in the proximal zone may migrate into this region. To determine whether proliferating *dbx1a:GFP*$^+$ cells specifically contribute to *de novo* neurogenesis after injury, we examined them as a subset of total HuC/D$^+$,BrdU$^+$ cells (Figs. 9M–O). A maximum of 2% of BrdU$^+$ cells in the rostral zone were neurons arising from the *dbx1a:GFP*$^+$ lineage, and levels in the rostral zone beyond 1dpi were not significantly different than controls. However, the neurogenic index of *dbx1a:GFP*$^+$ cells in the proximal zone was significantly greater than controls at 5dpi and 9dpi. In the blastema, there was no significant neurogenesis from *dbx1a:GFP*$^+$ cells until 9dpi. To determine the neurogenic contribution of *dbx1a:GFP*$^+$ cells to injury, we examined the percentage of newly born neurons arising from the *dbx1a:GFP* population. In the proximal zone, 74.40% ± 18.9% of newly born neurons were GFP$^+$ by 5dpi;

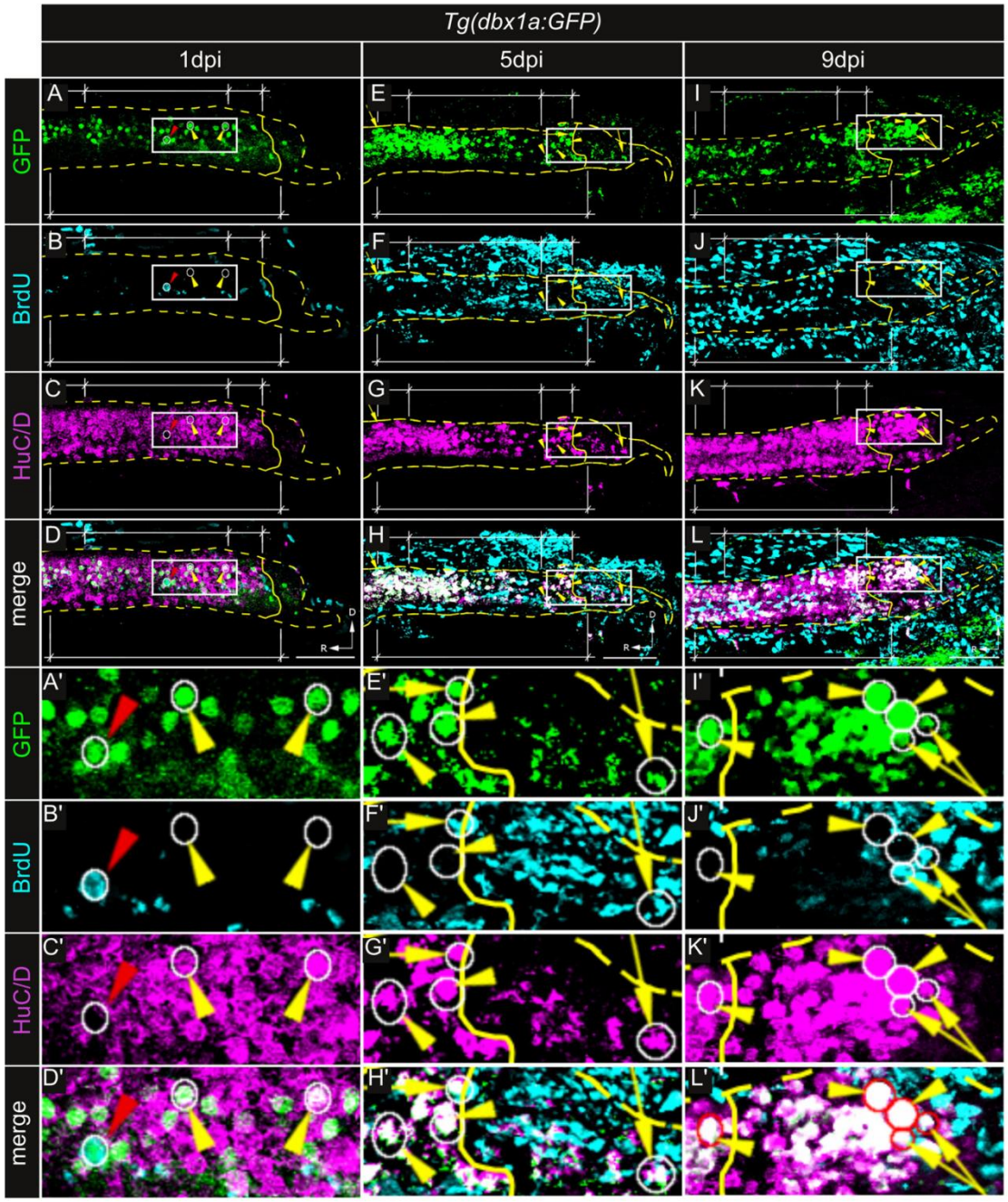
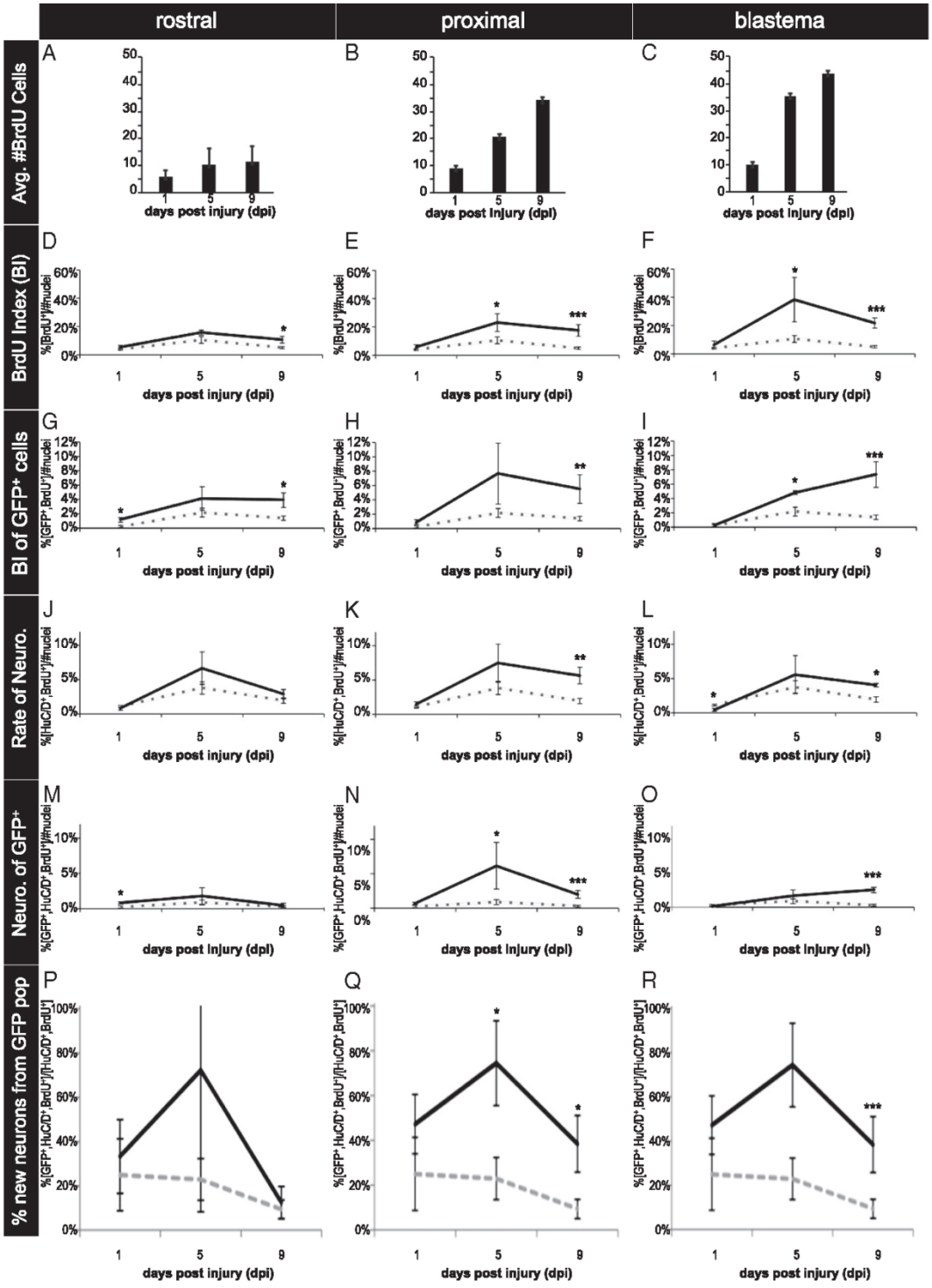


Fig. 8. *dbx1a:GFP*$^{+}$ cells exhibit a proliferative and neurogenic response to injury. (A–D) At 1dpi, [GFP^{+}, BrdU^{+}, Hu^{-}] cells (red arrowheads) are present in a healthy spinal cord (yellow dashed line), as are [GFP^{+}, Hu^{+}, BrdU^{-}] cells (yellow arrowheads); however, no GFP^{+} cells are present in the blastema (marked by solid yellow line). White box marks region shown in A'–D'. (E–H) At 5dpi, [GFP^{+}, Hu^{+}, BrdU^{+}] cells (arrows) representing neurons arising from the *dbx1a:GFP*$^{+}$ lineage born after injury are present in proximal and rostral zones; a few triple positive cells are present in the blastema as well. [GFP^{+}, Hu^{+}, BrdU^{-}] cells (arrowheads) are present in the proximal zone. White box marks region shown in E–H'. (I–L) At 9dpi, multiple [GFP^{+}, Hu^{+}, BrdU^{+}] (arrows) and [GFP^{+}, Hu^{+}, BrdU^{-}] (arrowheads) cells are present in the growing blastema; triple positive cells are observed at the proximal zone–blastema boundary but not in the rostral zone. White box marks region shown in I'–L'. Scalebar = 50 μm. All figures are Max-Z projections.


rostral
proximal
blastema
Avg. #BrdU Cells
BrdU Index (BI)
BI of GFP+ cells
Rate of Neuro.
Neuro. of GFP+
% new neurons from GFP pop
days post injury (dpi)

at 9dpi this percentage was reduced to 38.38% ± 12.64%. Interestingly, 63.04% ± 9.11% of newly born neurons in the blastema were GFP^+ at 9dpi (Figs. 9P–R). Together these data demonstrate that *dbx1a:GFP*$^+$ cells respond to injury by first rapidly expanding their progenitor pool throughout the rostral spinal cord with a subset of these new progenitors differentiating as neurons beginning at 5dpi, and that *dbx1a:GFP*$^+$ cells represent a neural progenitor population with a robust neurogenic response to spinal cord injury.

Discussion

dbx1a$^+$ *cells are neural progenitors*

In the embryonic mammalian spinal cord, *Dbx1* expression labels a multipotent progenitor population that gives rise to neurons, astrocytes and oligodendrocytes (Fogarty et al., 2005). Here, our data show that *dbx1a* expression identifies a progenitor population in the zebrafish spinal cord that persist as radial glia and give rise to neurons during embryogenesis. This suggests an evolutionary conservation of *Dbx1* expression between mammals and teleosts identifying neural progenitors of the intermediate spinal cord. We have also shown that at 5dpf, *dbx1a:GFP*$^+$ cells are separate from *olig2:dsRed*$^+$ cells, suggesting that these are two different progenitor populations. Previous analysis has shown that $Dbx1^+$ cells contribute to the V0 and V1 interneuron populations during amniote development (Pierani et al., 2001). However, in the absence of immunohistochemical markers to identify these interneurons, we have not yet been able to determine which neuronal subtypes *dbx1a:GFP*$^+$ cells produce during zebrafish development.

dbx1a:GFP$^+$ *cells persist as progenitors beyond embryogenesis*

In the mammalian spinal cord, $Dbx1^+$ cells are no longer detectable by E16.5 (Fogarty et al., 2005), indicating a loss of a multipotent progenitor population consistent with the observation that mammals cannot reinitiate neurogenesis following spinal cord injury. In contrast to mammals, *dbx1a* expression persists beyond embryogenesis until at least 14dpf in zebrafish, and *dbx1a:GFP* expression labels a slowly dividing neural progenitor population as evidenced by coexpression of GFAP, PCNA, and Sox3 with GFP. It remains to be shown whether *dbx1a:GFP*$^+$ progenitor cells persist into adulthood, and whether this marker identifies a neural stem cell population in the intermediate spinal cord at that time.

Larval zebrafish exhibit rapid and robust spinal cord regeneration

The adult zebrafish has been effectively established as a model for studying spinal cord regeneration (Becker et al., 2004; Goldshmit et al., 2012; Hui et al., 2010). Zebrafish take 2–4 months to reach sexual maturity, and coupled with a recovery time from spinal injury of 6–8wpi, we sought to establish a novel spinal cord injury model in the larval zebrafish that might represent shorter lead and recovery times and could also take advantage of genetic tools not available in the adult. We chose to transect the spinal cord at 5dpf because our data indicate that the peak of neurogenesis by *dbx1a:GFP*$^+$ cells occurs at this time, and beyond that point the progenitors become more quiescent. While there was variability in recovery from injury, we have demonstrated that larval zebrafish injured at 5dpf have neuronal soma in the injury site by 9dpi, compared to 4wpi in the adult (Hui et al., 2010). Additionally, sensory and motor function recovery in the larval zebrafish is complete by 9dpi, instead of the 6–8wpi observed in the adult zebrafish (Reimer et al., 2008). We therefore propose that the larval zebrafish is an effective model for studying spinal cord regeneration. While our data do not conclusively prove that functional recovery in larvae requires neurogenesis or axon regrowth, the timing of these three events is well correlated.

dbx1a$^+$ *cells contribute to a proliferative and neurogenic response during spinal cord regeneration*

By using BrdU to label proliferative cells before spinal cord transection, we were able to track equivalent populations in injured and control animals, and monitor their proliferation. Our data show that the proliferative response to injury is systemically present by 5dpi, but is more pronounced in the region proximal to the injury site and in the regenerating blastema. When we examined *dbx1a:GFP*$^+$ cells as a subset of the $BrdU^+$ population, we observed similar dynamics with a higher relative increase in proliferation compared to uninjured controls. We therefore conclude that spinal cord transection results in a rapid proliferative response from existing mitotic progenitors, including *dbx1a:GFP*$^+$ cells.

BrdU labeling before injury also allowed us to trace the differentiation of dividing progenitors into neurons. When examining all $BrdU^+$ cells, we found that the significant neurogenic response to injury was restricted to the region proximal to the injury site and the blastema, unlike the more widespread proliferative response. These data suggest that more rostral progenitors which proliferate following injury may die, remain undifferentiated, or migrate prior to differentiation. Significant levels of newly born neurons did not accumulate until after 5dpi, showing evidence of a delay between the proliferative and neurogenic responses, and the subset of *dbx1a:GFP*$^+$ cells labeled by BrdU showed similar dynamics, indicating that the transgene marks neural progenitors contributing to repair following injury. Furthermore, our analysis suggests that most neurons born after injury arise from the *dbx1a:GFP*$^+$ progenitor population. Our experiments do not allow us to distinguish between the possibilities that progenitors differentiate directly in the blastema, or that newly born *dbx1a:GFP*$^+$ neurons migrate to the blastema from an uninjured spinal cord. Regardless, it is likely that migration of either progenitors or neurons occurs because the blastema region is almost entirely devoid of cells following transection.

In conclusion, we have shown that *dbx1a:GFP* expression marks a pool of neurogenic spinal radial glial progenitors that persist beyond the end of embryogenesis, and contribute to the regenerative response by proliferating and subsequently differentiating as neurons. Based on their multipotency, longevity, slow rate of proliferation in the absence of injury and their rapid regenerative response, our data indicate that *dbx1a:GFP*$^+$ radial glia are likely stem cells for the regeneration of interneurons following spinal cord injury in zebrafish.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.expneurol.2014.03.017.

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Fig. 9. Time course of proliferative and neurogenic responses following transection. (A–C) The number of $BrdU^+$ cells following injury remains relatively unchanged in the rostral region during recovery (A), but a steady increase is observed in proximal (B) and blastema (C) zones. (D–F) Significant increases in the BrdU labeling index are observed in proximal and blastema zones by 5dpi. (G–I) The index of $BrdU^+$,*dbx1a:GFP*$^+$ cells also increases following injury, with a large number of $BrdU^+$,GFP^+ cells appearing in the blastema by 9dpi. (J–L) Significant accumulations of newly-born ($BrdU^+$) neurons are observed in proximal and blastema zones by 9dpi. (M–O) A significant increase in newly-born GFP^+ neurons is observed in the proximal zone by 5dpi, and in the blastema by 9dpi. (P–R) Percentage of newly born neurons arising from *dbx1a:GFP*$^+$ population. Dashed line represents sham data, black lines are transected animals. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; n = 5–7 animals per condition, per timepoint for each set of experiments; error bars = SEM.

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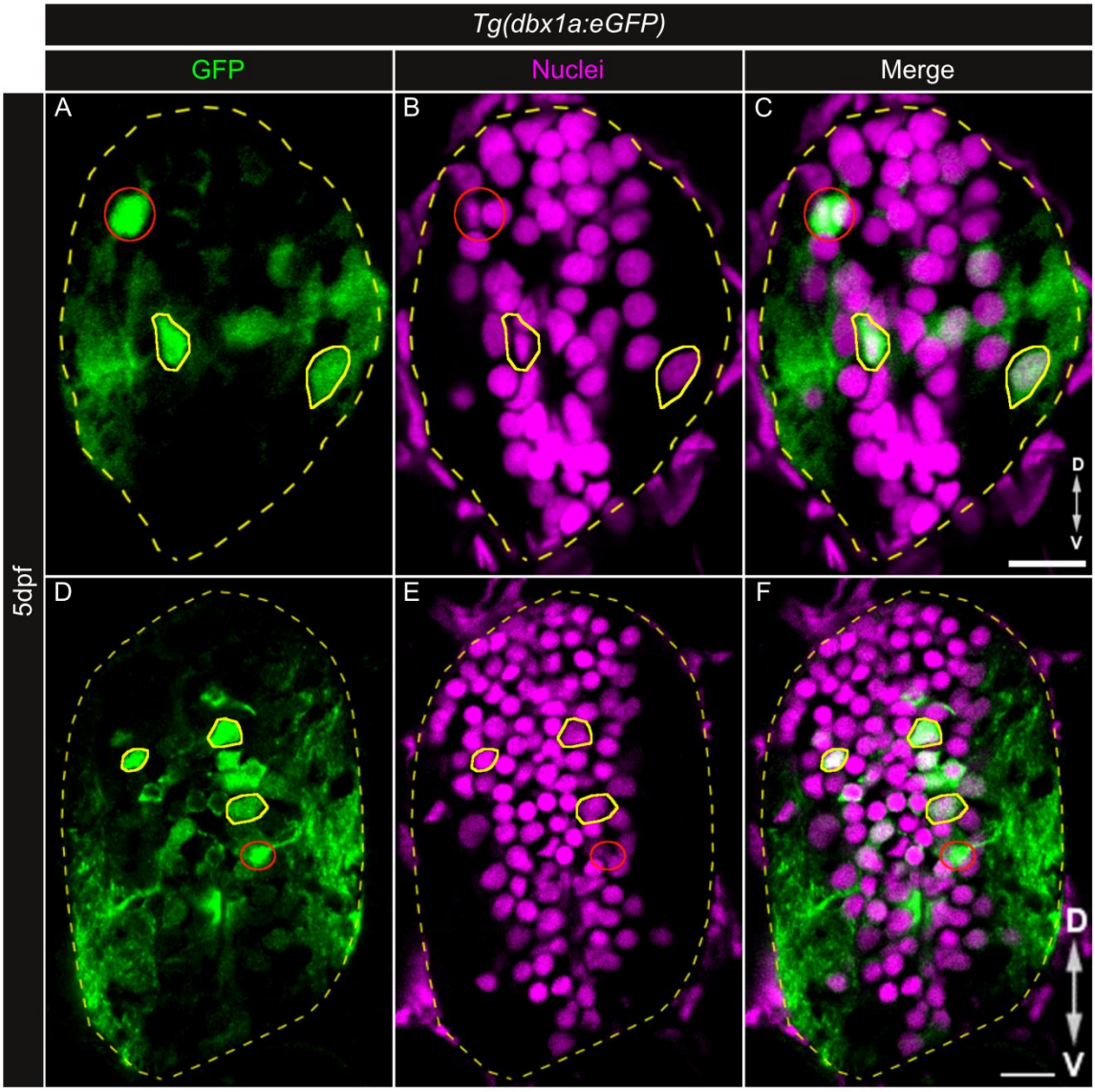
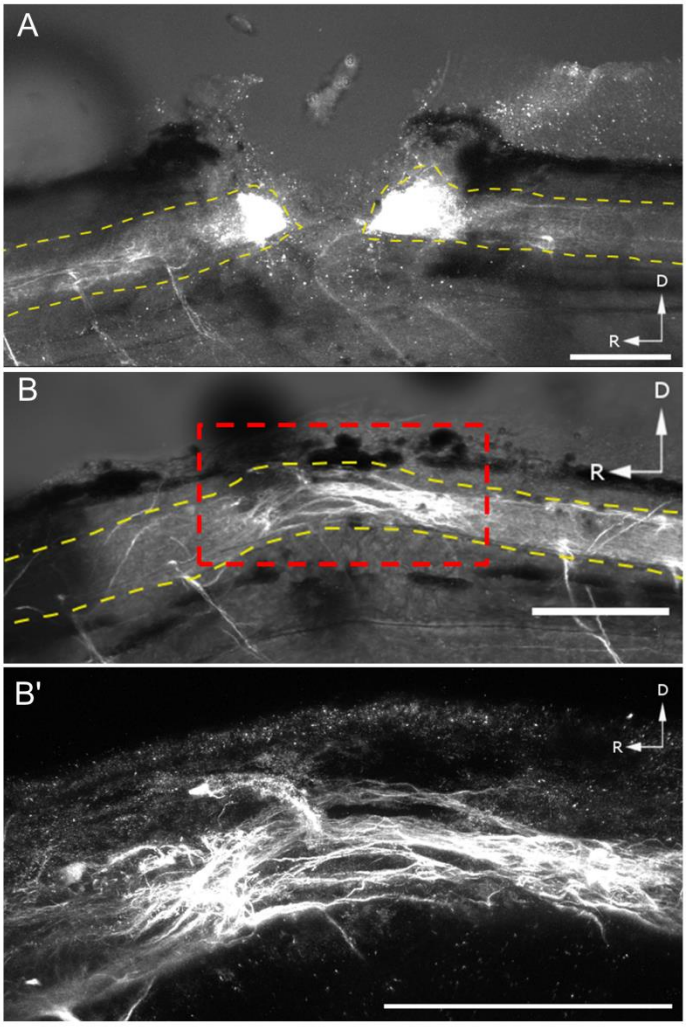
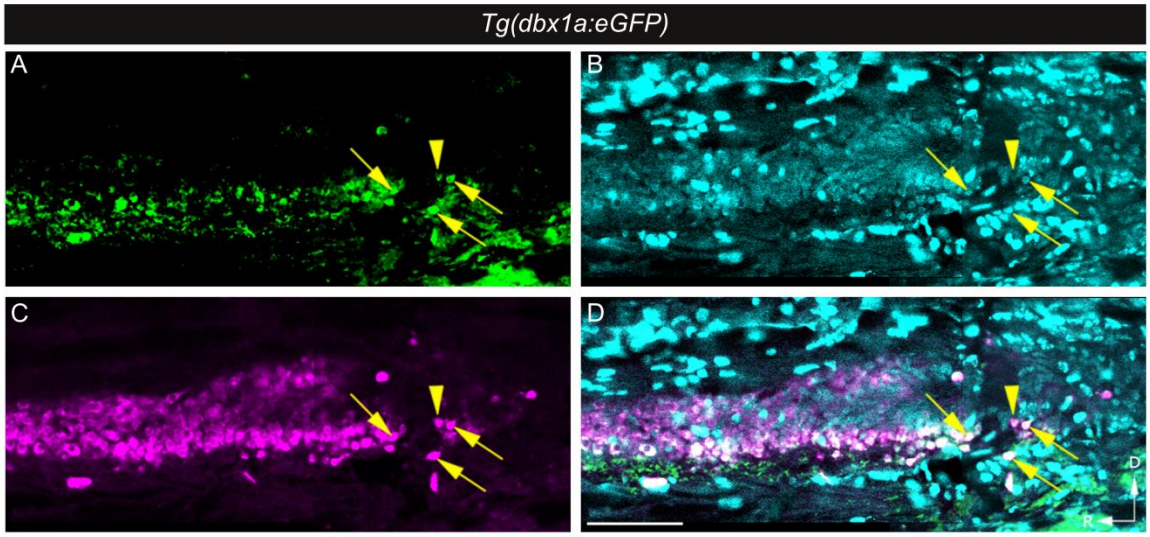


Supplementary Fig. 1.
Identification of GFP⁺ cells suitable for analysis. Cells outlined in yellow are GFP⁺ based on colocalization of GFP expression with nuclear staining. Cells outlined in red are GFP⁺ but lack a nucleus in this particular section, and would thus not be considered a GFP⁺ cell suitable for analysis. (A–C) Compare with Fig. 2A. (D–F) Compare with Fig. 2L. Scale bars = 10 μm; all panels are single optical sections.



Supplementary Fig. 2.
Axons cross the injury gap as part of the regenerative response. (A) Using anti-acetylated tubulin to label axons, there is a clear gap between spinal cord stumps with no axons crossing the injury site at 1dpi. (B) At 5dpi, multiple axons have crossed the injury site; red box marks region shown in B'. Scale bars = 100 μm; all figures are Max-Z projections.



Supplementary Fig. 3.
Single confocal slice analysis of neurogenesis at 9dpi. (A–D) GFP^{+}, HuC/D^{+} and $BrdU^{+}$ cells (arrows) are present in the blastema, as are $BrdU^{-}$ neurons (arrowhead). Scalebar = 50 μm.

CHAPTER 4

WNT/ß-CATENIN SIGNALING IS REQUIRED FOR THE NEUROGENIC RESPONSE FOLLOWING SPINAL CORD TRANSECTION IN LARVAL ZEBRAFISH

Abstract

Spinal cord injury results in permanent sensory-motor loss in mammals, in part due to a lack of injury-induced neurogenesis. Postembryonic neurogenesis depends upon a resident neural progenitor population, which in zebrafish persist as a subset of radial glia. However, the molecular mechanisms regulating these progenitors remain uncharacterized. Wnt/ß-catenin signaling is necessary for the regenerative response of multiple tissues, including the zebrafish fin. However, it is not known whether spinal cord regeneration requires Wnt signaling as well. Here we show that spinal radial glia exhibit Wnt/ß-catenin activity as they undergo neurogenesis following transection. We then use Cre-mediated lineage tracing to label the progeny of radial glia and show that Wnt/ß-catenin signaling is required for progenitors to fully differentiate into neurons. Our data thus establish Wnt/ß-catenin pathway activation as a necessary step in spinal cord regeneration.

Introduction

Spinal cord injury (SCI) is a permanent, chronic condition in humans, representing significant economic and quality of life burdens (National Spinal Cord Injury Statistical Center, 2013). However, SCI is not permanent in other vertebrates such as the zebrafish *Danio rerio* (Becker et al., 1997; Kuscha et al., 2012). The remarkable recovery of sensory and motor function observed even after complete spinal

cord transection involves two companion paradigms of regeneration: axonal regrowth and *de novo* neurogenesis. Restoration of motor- and interneuron populations after injury rely upon activation of local neural progenitor cells (NPCs; Briona and Dorsky, 2014; Reimer et al., 2008). In zebrafish, NPCs persist beyond embryogenesis as a subpopulation of radial glia (Johnson et al., 2014; Kizil et al., 2012). During development, NPC proliferation and maintenance is dependent upon the Wnt/ß-catenin pathway (Bonner et al., 2008; Gan et al., 2014; Wang et al., 2012). In regenerative animal injury models, Wnt/ß-catenin signaling is upregulated in the regeneration blastema, and inhibition of Wnt/ß-catenin after injury arrests the regenerative process (Stoick-Cooper et al., 2007; Yokoyama et al., 2007). Following zebrafish caudal fin amputation, surviving terminally-differentiated cells dedifferentiate, proliferate, and re-differentiate to regenerate lost tissue(Stewart and Stankunas, 2012). Recently, it was shown that canonical Wnt signaling governed blastemal cell proliferation and osteoblast differentiation in the regenerating zebrafish caudal fin (Wehner et al., 2014). Unlike the dedifferentiate-proliferate-redifferentiate paradigm of regeneration observed in zebrafish tail fin amputation, regeneration following spinal cord injury relies upon asymmetrical proliferation of local neural progenitor cells for recovery (Hui et al., 2010; Reimer et al., 2008). Whether Wnt/ß-catenin signaling regulates injury-induced neurogenesis following SCI as part of the regenerative process is not known.

To identify and characterize the behavior of radial glia following SCI, we generated a stable GFAP:Cre^{ERt2} zebrafish line that shows rapid 4-OHT-inducible Cre activity. We show here that a subpopulation of converted cells are GFAP^{+} radial glia that are minimally neurogenic during development, but that converted cells increase proliferation and neurogenesis in response to SCI in the larval zebrafish. We also show that Wnt/ß-catenin activity is present in radial glia following transection, and that when signaling is pharmacologically inhibited after SCI, there is a reduction of neurogenesis by

converted NPCs. Together, our data suggest that Wnt/ß-catenin signaling is required for the neurogenic response to SCI.

Materials and methods

Fish strains and staging

Embryos were obtained from wildtype (AB*), *Tg(7xTCF-Xla.Siam:GFP)*^{ia4} (*7xTCF:GFP)*, and *Tg(ubi:loxP-eGFP-loxP-mCherry)*^{cz1701} (*ubi:Switch)*, and staged according to Kimmel et al. (1995). Zebrafish were raised and bred in the University of Utah core facility Centralized Zebrafish Animal Resource according to standard procedures; experiments were approved by the University of Utah Institutional Animal Care and Use Committee.

Vectors and transgenesis

p5E-GFAP and *pME-Cre*^{ERt2} were generous gifts from Fabienne Poulain; destination vector *pDestTol2cryYFP-pA* was a kind gift from Christian Mosimann; *p3E-polyA* is Tol2kit vector #302, previously described (Kwan et al., 2007).

Tg(GFAP:Cre^{ERt2}*,cry:YFP)*^{zd16} is a Multisite Gateway assembly of *p5E-gfap*, *pME-cre*^{Ert2}, and *p3E-polyA*, made according to standard protocols (Kwan et al., 2007), using LR Clonase II Plus (Invitrogen #12538-120).

Plasmid DNA for injection was purified using HiSpeed Plasmid Midi Kit (Qiagen). To generate transgenic zebrafish lines, 30ng/µl plasmid DNA and 25ng/µl transposase RNA were co-injected into 1-cell stage wildtype embryos using a PLI-100 microinjector (Harvard Apparatus). Embryos were screened for αcrystallin-YFP expression and raised to adulthood, when they were crossed with wildtype animals to screen for germline transmission. Four founders were identified.

4-OHT treatment for cre^{ERt2} induction

Tg(GFAP:cre^{ERt2},cry:YFP)^{zd16} and Tg(*ubi:Switch)* animals were crossed, embryos [*Tg(GFAPubi)*] screened for robust GFP expression, and raised in recovery media (E2 media + 0.2mM phenylthiourea (Sigma) + 10mg/L gentamycin sulfate (Amresco)) at 28.5°C. At 4 days postfertilization (dpf), larvae were transferred to fresh recovery media containing 10mM BrdU (Sigma) and 5µM 4-hydroxytaxmoxifen (4-OHT, Sigma) for 24 hours in the dark prior to surgery.

Spinal cord injury

Larvae were lesioned at 5dpf as described previously (Briona and Dorsky, 2013, 2014). Briefly, microinjection pipettes were broken, beveled, and used as glass scalpels. Larvae were anesthetized with 0.016% Tricaine (Sigma), and their spinal cords transected at the level of the anal pore with a glass scalpel. Injured animals were allowed to recover in recovery media for 6 hours before commencement of Wnt inhibition studies.

Inhibition of Wnt/ß-catenin signaling

Animals were transferred to recovery media containing 35µM IWR1 (Sigma, #IO161-25MG). Media was made fresh and changed daily after feeding.

Immunohistochemistry

Larvae intended for cryosectioning before immunohistochemistry were fixed in fresh 4% PFA for 1 hour at room temperature, washed in PBS, cryoprotected in sucrose, embedded in OCT, and sectioned at 50µm thickness on a Leica CM3050 as described previously (Briona and Dorsky, 2014). Larvae intended for whole mount immunohistochemistry were fixed overnight in fresh 4% PFA at 4°C, equilibrated in PTW, heat treated in 70°C Tris-HCl pH 9.0, and dehydrated with acetone as described

previously (Inoue and Wittbrodt, 2011). Primary antibodies used were: Rabbit anti-GFP (1:5000, Invitrogen #A-11122), chicken anti-GFP (1:1000, Aves #GFP-1020), mouse anti-HuC/D (1:500, Invitrogen #A-21271), chicken anti-BrdU (1:500, ICL #CBDU-65A-Z), mouse anti-PCNA (1:1000, Sigma #p8825), rabbit anti-Sox3 (1:200, Pierce Custom Antibodies and Peptides), rabbit anti-DsRed (1:200, Clontech #632496), and mouse anti-GFAP (1:200, Zebrafish International Resource Center #zrf1). Secondary antibodies used were: goat anti-rabbit 488 (1:200, Invitrogen #A-11008), goat anti-rabbit 568 (1:200, Invitrogen #A-11041), goat anti-rabbit cy3 (1:200, Jackson ImmunoResearch #111-165-003), goat anti-mouse 633 (1:200, Invitrogen #A-21050), goat anti-mouse cy3 (1:200, Jackson ImmunoResearch #115-165-003), goat anti-chicken 488 (1:200, Invitrogen #A-11039), and goat anti-chicken 633 (1:200, Invitrogen #A-21103). Hoechst 33342 was used to visualize nuclei.

Confocal microscopy

Larvae or sections were imaged using an Olympus FV-1000XY or Nikon A1 confocal microscope housed in the University of Utah Cell Imaging Core Facility. Images were processed using ImageJ (rsbweb.nih.gov/ij) and GIMP (gimp.org).

Quantification

Analysis based on cryosection of uninjured fish used five nonconsecutive transverse sections from five embryos per timepoint; analysis based on injured fish used five fish per timepoint. Antibody colocalization was scored using MaxZ projections generated with ImageJ and verified using single slices.

Statistical analysis

All results were expressed as mean ± SEM. Two-tailed two-sample equal variance Student t-tests were calculated using Excel; differences were considered

significant at $p<0.05$.

Results and discussion

Wnt reporter expression is upregulated in response to SCI

Canonical *Wnt* gene expression is detected at the leading edge of the blastema in the regenerating zebrafish caudal fin as early as 6 hours postamputation (hpa), while Wnt reporter activity is detectable by 12hpa (Stoick-Cooper et al., 2007; Wehner et al., 2014). Additionally, in the regenerating *Xenopus* limb bud, *Xwnt3a* is detectable in the blastema by 3 days postamputation (Yokoyama et al., 2007). Inhibition of Wnt/ß-catenin signaling in the regenerating tail fin or limb bud halts blastemal proliferation (Wehner et al., 2014; Yokoyama et al., 2007), Together, these data suggest that canonical Wnt signaling may be generally required for the proliferative response following injury in regenerative animals. To determine whether Wnt/ß-catenin signaling was active during regeneration after SCI, we injured *Tg(7xTCF:GFP)* larvae and examined GFP expression daily from 1-9dpi; as we have previously shown that following spinal transection in the larval zebrafish, severed ends of the spinal cord rejoined within 9 days postinjury (dpi; Briona and Dorsky, 2014, Figs. 4.1A-E). In uninjured spinal cord, we found that reporter expression was completely absent (not shown). In contrast, GFP^+ cells with radial glial-like morphology were observed in the blastema until 7dpi, suggesting Wnt activity is involved in the spinal regenerative process, and that the active window of analysis to further investigate the role of Wnt activity following injury is 1-7dpi.

To determine which cells express the Wnt reporter, we examined GFP and cell-specific marker colocalization: GFAP to label radial glia, Sox3 to identify neural precursors, and HuC/D to identify neurons. 18% ± 9.96% of GFP^+ cells were $GFAP^+$ radial glia at 1-3dpi; this increased to 31% ± 7.54% by 5dpi (Fig. 4.1N). $Sox3^+$ Wnt-

reporter expressing cells were detectable at 1dpi (data not shown). At 1dpi, 9.08% ± 3.23% of GFP$^+$ were Sox3$^+$; this proportion increased to 19.27% ± 6.02% at 3dpi, and at 7dpi, 28.42% ± 2.25% of neural progenitors present also expressed the Wnt-reporter. Neural precursors arising from GFP-expressing radial glia were observed in the blastema as early as 3dpi (Figs. 4.1F-I, O), with 11.51% ± 7.28% Wnt-reporter-expressing cells positive for both GFAP and Sox3 expression. This proportion decreased slightly but insignificantly at 5dpi to 10.16% ± 6.80%. GFP$^+$ neurons were detected by 5dpi (Figs. 4.1J-M, P).

These data show that Wnt signaling is upregulated in response to spinal transection in the blastema, suggesting that Wnt/ß-catenin signaling is an evolutionarily conserved response to injury, not just amputation (Stoick-Cooper et al., 2007). We have also shown that approximately 20% of the reporter-expressing cells in the blastema are radial glia, and that most of these radial glia are Sox3$^+$ neural progenitors. These data suggest that Wnt/ß-catenin signaling is involved in the regenerative response to SCI.

Tg(GFAP:Cre^{ERt2}, cry:YFP)^{zd16} labels neurogenic spinal radial glia

Neural progenitors in the zebrafish CNS persist as radial glia (Johnson et al., 2014; Kizil et al., 2012). To examine the neurogenic capacity of radial glia following SCI, we generated a stable transgenic line with Cre^{ERt2} expressed under the zebrafish *gfap* promoter: *Tg(GFAP:Cre^{ERt2}, cry:YFP)*. When progeny from a cross to *Tg(ubi:Switch)* fish were exposed to tamoxifen, *Tg(GFAP:Cre^{ERt2},cry:YFP; ubi:Switch)* (Mosimann et al., 2011) double-positive embryos (hereafter referred to as *GFAPubi*) expressed mosaic mCherry in the spinal cord within 24 hours of exposure, thereby permanently labeling radial glia and their progeny.

To characterize the behavior and identify the lineage of converted *GFAPubi* cells, we treated 4dpf embryos with 5µM 4-OHT for 24 hours, then raised them until 14dpf. At

6dpf, 76% ± 3.42% of converted *GFAPubi* cells were GFAP$^+$ based on zrf1 antibody staining (Figs. 4.2A-C). The proportion of GFAP$^+$ converted cells stayed relatively consistent until 14dpf, by which time it had decreased to 61% ± 3.22% (Fig. 4.2D).

While *GFAPubi* converted cells contribute to the neuronal population by 10dpf, and 4.68% ± 1.25% converted cells expressing HuC/D were detected at 14dpf (Figs. 4.2E-H), the low rate of neurogenesis indicates that *GFAPubi* labels a relatively quiescent population. To measure proliferative activity, we incubated *GFAPubi* embryos with 5μM 4-OHT and 10mM BrdU from 4-5dpf, then examined BrdU incorporation. Between 6-14dpf, the percentage of BrdU-labeled GFAPubi cells remained consistent, with an eventual decrease (Figs. 4.2I-L). Together, these data indicate that radial glia undergo few cell divisions and do not generate many neurons in the uninjured spinal cord.

These data show that most lineage-labeled cells persist long-term as GFAP$^+$ radial glia, and that some of these cells are capable of contributing to the spinal neuronal population. In addition, we conclude that our *Tg(GFAP:Cre^{ERt2}, cry:YFP)* line is an effective new addition to the zebrafish genetic toolbox for studying radial glia.

Converted GFAPubi cells in the blastema proliferate and make neurons in response to SCI

We next asked what contribution *GFAPubi* converted cells made to the regenerative process after SCI. We incubated GFAPubi fish in 5μM 4-OHT for the 24 hours immediately preceding spinal cord transection at 5dpf to mosaically label spinal radial glia and their progeny. At 1dpi, 96.67% ± 3.33% of converted cells in the blastema were GFAP$^+$ based on zrf1 antibody staining. By 3dpi, only 32.52% ± 5.47% converted cells were GFAP$^+$. This decreasing trend continued until 5dpi, where merely 19.36% ± 4.90% converted cells were GFAP$^+$ (Figs. 4.3A-C, G). The reduction in GFAP$^+$ converted cells in the blastema could be due to cell death if the population of converted cells

remained quiescent after SCI. However, at 1dpi, 6.85% ± 0.26% of cells in the blastema were converted (mCherry$^+$). This proportion increased to 22.97% ± 6.19% at 3dpi, and at 7dpi, 29.17% ± 7.55% of cells in the blastema expressed mCherry, suggesting that converted cells proliferate and differentiate in response to injury rather than remaining quiescent.

To determine the neurogenic potential of *GFAPubi* converted cells, we examined mCherry and HuC/D colocalization after injury. mCherry$^+$ neurons were observed in the blastema beginning 3dpi, where 34.85% ± 11.45% converted cells were HuC/D$^+$. The proportion of mCherry$^+$ neurons slowly increased over the duration of the experiment, with 42.76% ± 3.91% of converted cells expressing the neuronal marker at 7dpi (Figs. 4.3D-F, H). In contrast to their minimally neurogenic nature during development, mCherry$^+$ neurons represent 45.58% ± 4.71% of neurons in the blastema at 7dpi.

Together these data show that *GFAPubi* labels a quiescent spinal neural progenitor population (NPCs) that exhibit a proliferative and neurogenic response to injury.

Treatment with the Wnt inhibitor IWR1 after injury blocks Wnt activity but does not affect the endogenous rate of proliferation in uninjured gut and spinal cord

Before using the small molecule inhibitor of Wnt response (IWR1) to test the role of Wnt/ß-catenin signaling in spinal cord regeneration, we first confirmed that a dose of 35µM IWR1 was sufficient to inhibit Wnt-reporter expression in the blastema after SCI without being lethal (Fig. 4.4). Low levels of canonical Wnt signaling are required for homeostatic proliferation (Bernascone and Martin-Belmonte, 2013; van Es et al., 2012). To verify that any IWR1-related observations after SCI were specific to the regenerative process, we next incubated uninjured 4dpf wildtype larvae in 10mM BrdU for 24 hours,

then transferred them to media containing 0μM or 35μM IWR1 for 2 days (short pulse) or 9 days (long pulse). We examined gut (rapid turnover of tissue) and spinal cord (target tissue with slow rate of turnover) for BrdU incorporation and found that there was no significant difference between control and IWR1-treated animals at either timepoint examined (Fig. 4.5). We therefore conclude that 35μM IWR1 is sufficient to inhibit Wnt signaling in response to injury, and that this concentration does not affect proliferation in uninjured tissue.

IWR1 inhibits the neurogenic response of converted GFAPubi cells to SCI

To determine whether Wnt/ß-catenin pathway signaling is required for spinal cord regeneration, we transferred injured *GFAPubi* larvae to recovery media containing 35μM IWR1 6 hours after injury, as immersion in this IWR1-media immediately after injury resulted in 100% mortality by 18 hours postinjury (data not shown).

No difference in the percentage of $GFAP^+$ converted cells with or without IWR1 was detectable at 1dpi; however, there was a significantly greater percentage of converted cells in the blastema of IWR1-treated larvae expressing GFAP at 3dpi and 5dpi compared to controls: 64.29% ± 12.02% vs. 32.52% ± 5.46% (3dpi) and 70.65% ± 8.33% vs. 19.36% ± 4.89% at 5dpi, respectively (Figs. 4.6A-C, G; compare with Figure 4.3), suggesting that in the presence of IWR1, radial glial progenitors may be unable to differentiate as neurons and instead accumulate in the blastema as NPCs.

Examination of $mCherry^+$ neurons in the blastema confirmed that fewer neurons were made between 3-5dpi in the presence of IWR1, with a significant decrease observed at 5dpi: only 13.33% ± 7.30% $mCherry^+$ cells expressed HuC/D (Figs 4.6D-F, H; compare with Figure 4.3). We have shown previously that the peak of neurogenesis following SCI occurs at 5dpi (Briona and Dorsky, 2014); to determine whether Wnt/ß-catenin signaling is required for the neurogenic response to injury after this timepoint, we

allowed injured *GFAPubi* animals to recover in recovery media until 5dpi, then transferred them to recovery media + IWR1 from 5-7dpi and examined coexpression of mCherry and HuC/D. No significant difference in the proportion of red neurons in the blastema was observed (Fig. 4.6H), suggesting that the critical window of Wnt/ß-catenin signaling for neurogenesis following SCI is 2-5dpi.

Discussion

We present here a new transgenic line *Tg(GFAP:Cre^{ERt2})* that permanently labels radial glia under temporal control, representing a significant addition to the zebrafish genetic tool box. In addition to studying the glial response to injury, this line can be used for glial fate mapping in other CNS tissues. Using this new line, we have identified a quiescent GFAP^{+} spinal radial glia population that exhibits prolific neurogenesis in response to injury, suggesting that there are bonafide neural stem cells in the larval spinal cord. At this point, we do not know whether this population is similarly active in the adult zebrafish.

Our data clearly indicate that Wnt/ß-catenin activity, which is normally absent in the uninjured spinal cord, is rapidly induced in radial glial neural progenitors following SCI, and persists throughout the regeneration process. Using pharmacological inhibition, we have also shown that Wnt/ß-catenin signaling is necessary for injury-induced neurogenesis in a stem-cell-based model of spinal cord regeneration, suggesting that this pathway has a conserved role in multiple regenerative responses. Together these experiments lead to a model in which extrinsic Wnt signals are induced after spinal cord injury, and act directly on radial glia to promote their differentiation into neurons. Identifying downstream targets of canonical Wnt signaling involved in the neurogenic response will be informative in the pursuit of sensory and functional recovery after SCI in humans.

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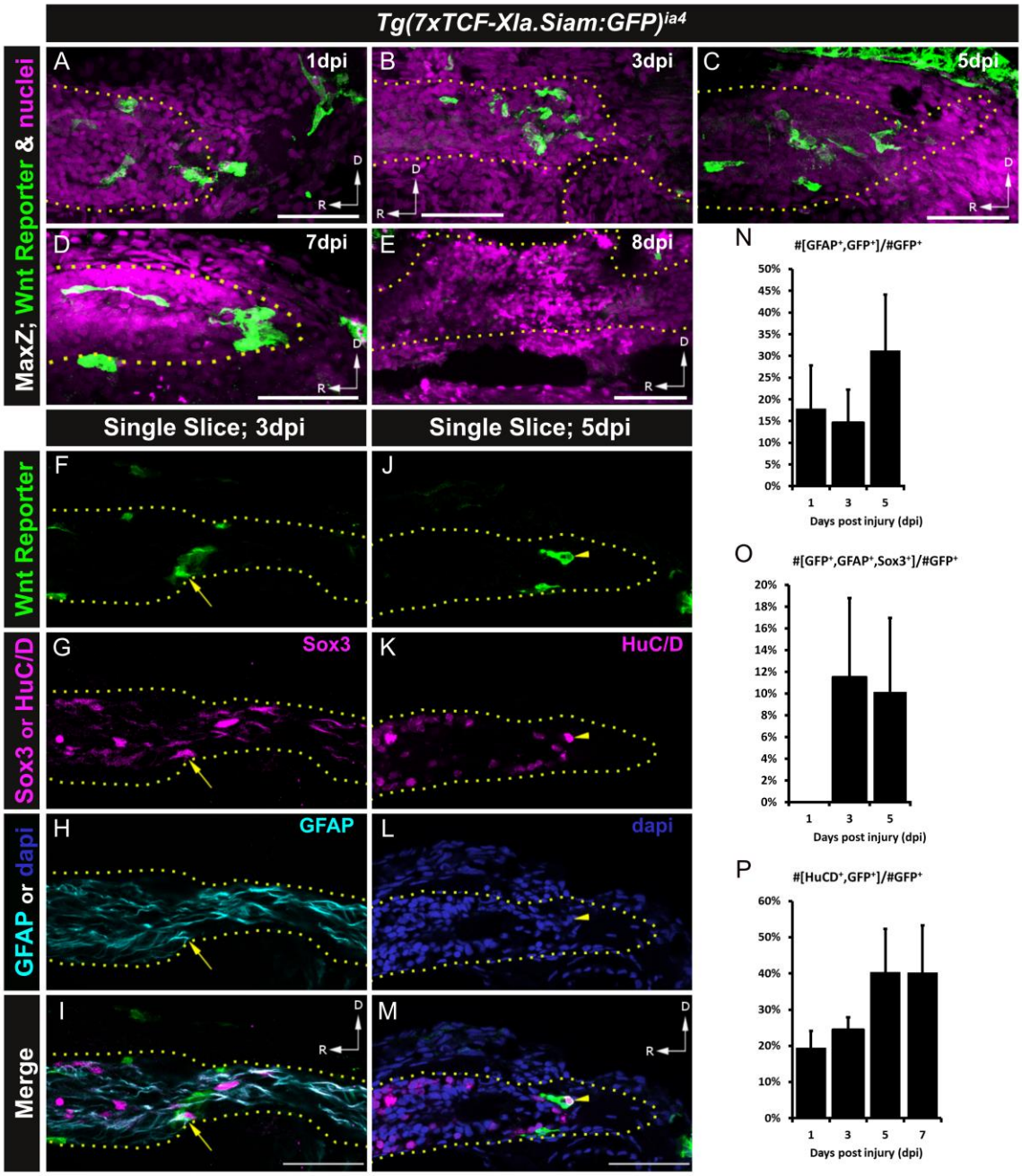

Figure 4.1: Wnt reporter expression in the blastema after SCI. (A-E) Time course of Wnt reporter expression after SCI shows pathway activity from 1-7dpi. (F-I) Wnt reporter-expressing cells are radial glial neural progenitor cells (arrow). (J-M) Wnt reporter-expressing cells make neurons (arrowhead). (N) Percentage of Wnt reporter cells expressing radial glia marker GFAP. (O) Percentage of Wnt reporter-positive radial glia expressing neural progenitor marker Sox3. (P) Percentage of Wnt reporter cells expressing pan-neuronal marker HuC/D. n=5 at each timepoint; error bars = SEM; scalebar = 50μm.

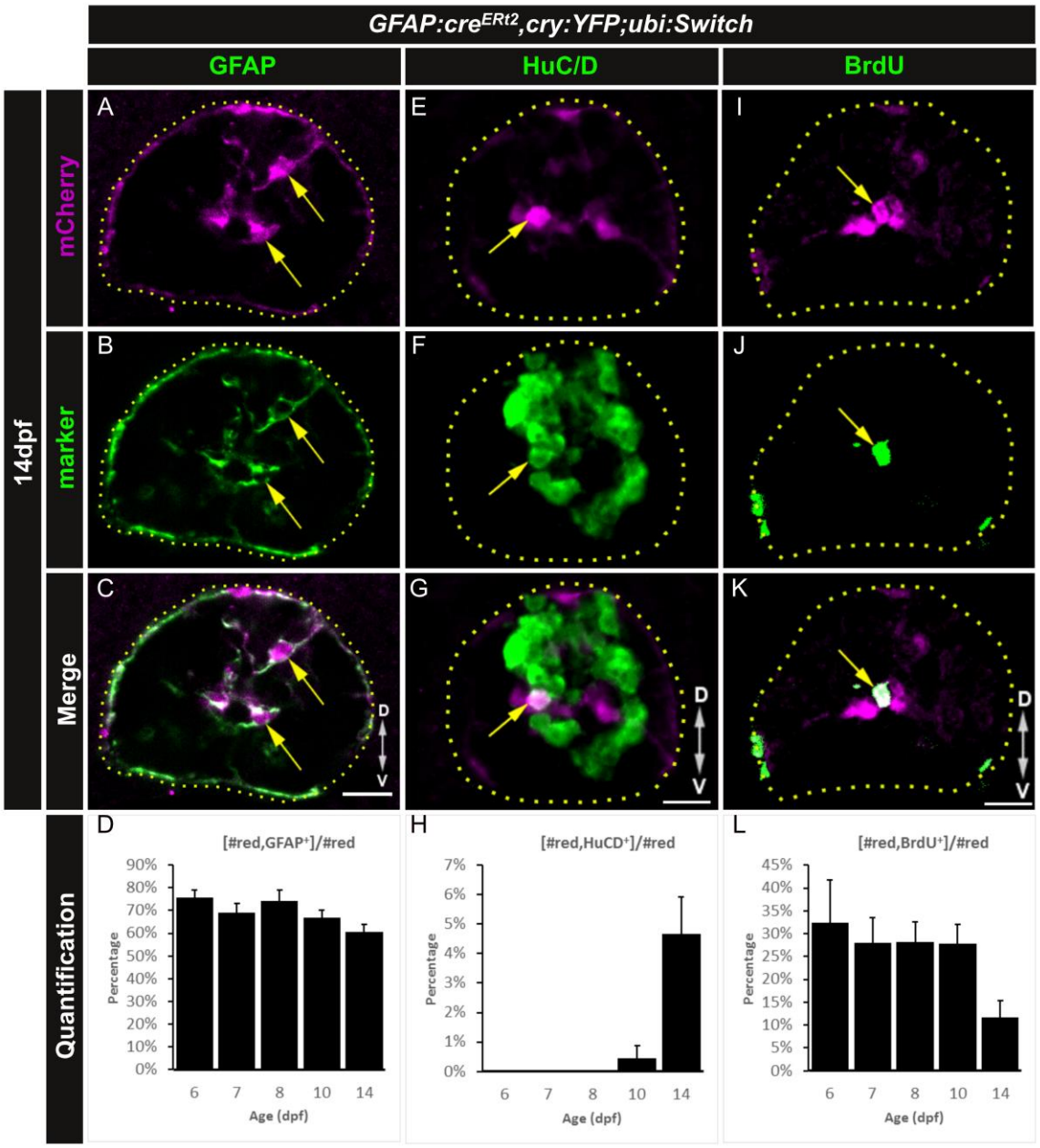

Figure 4.2: *Tg(GFAP:cre^{ERt2}, cry:YFP)* labels radial glia. (A-C) a GFAP antibody colabels converted cells. (D) percentage of converted cells that express GFAP. (E-G) converted cells make neurons. (H) percentage of converted cells expressing HuC/D. (I-K) converted cells are quiescent. (L) percentage of converted cells incorporating BrdU. n=25 at each timepoint; error bars = SEM; scalebar = 10μM.

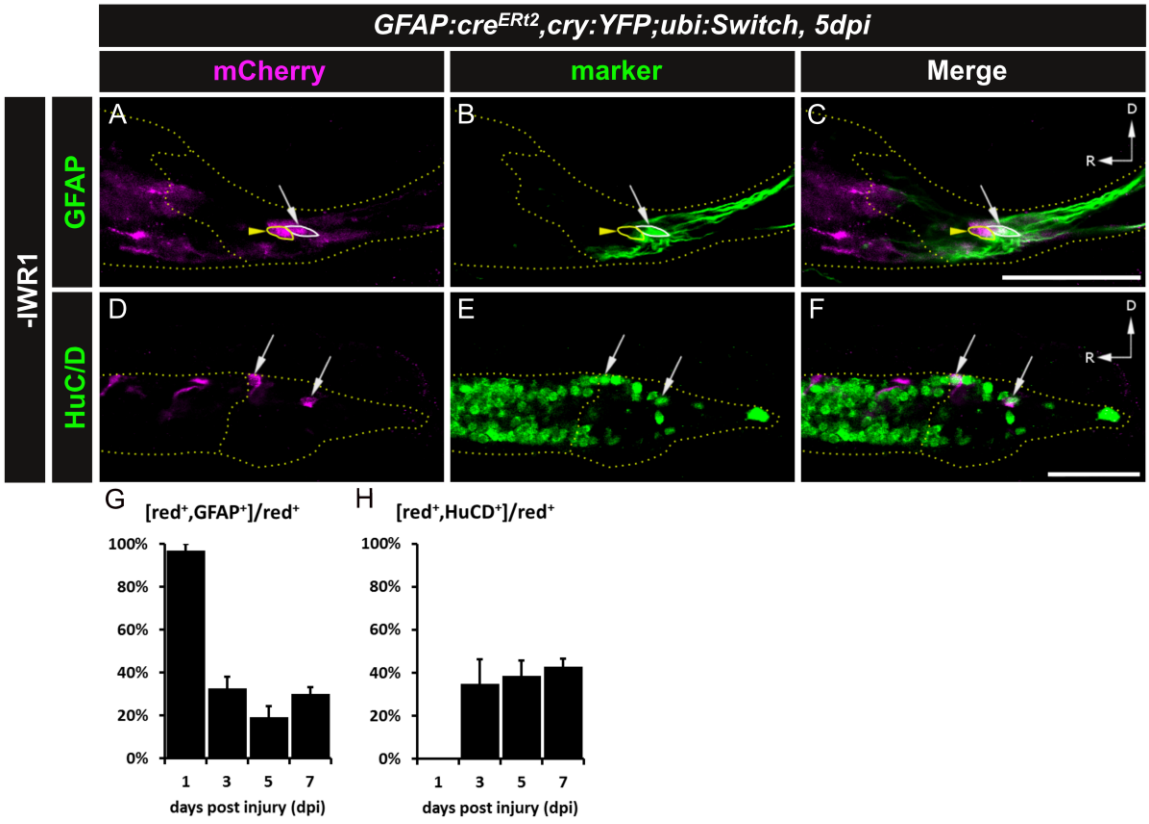

Figure 4.3: GFAPubi cells are neurogenic in response to injury. (A-C) converted cells in the blastema are radial glia. (D-F) Converted cells express HuC/D in the blastema. (G) Quantification of converted radial glia in the blastema. (H) Quantification of converted neurons in the blastema. n=5 at each timepont; error bars = SEM, scalebar = 50um.

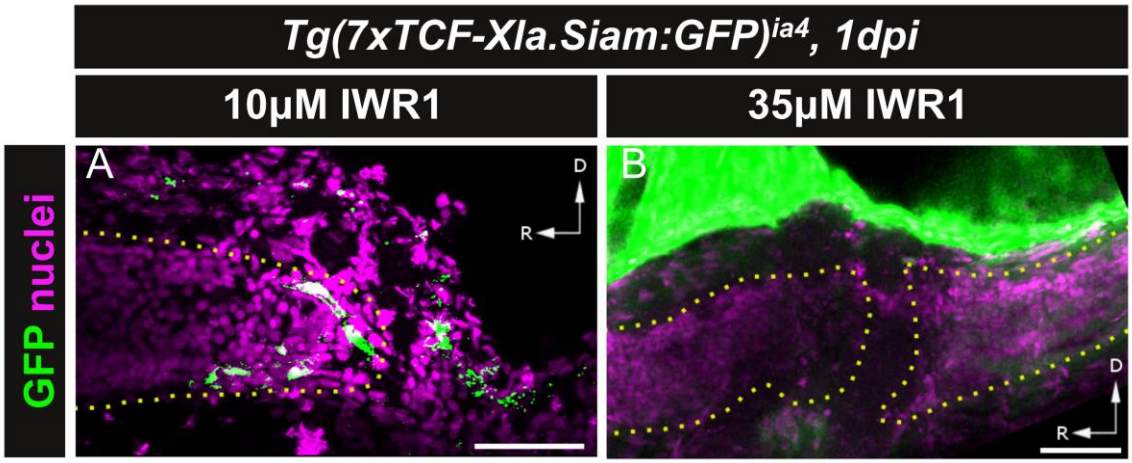

Figure 4.4: 35µM IWR1 is sufficient to inhibit Wnt-reporter expression after SCI. (A) 10µM is insufficient (compare with Figure 4.1A). (B) At 35µM, Wnt-reporter expression is absent from the blastema. n=15; scalebar = 50µm.

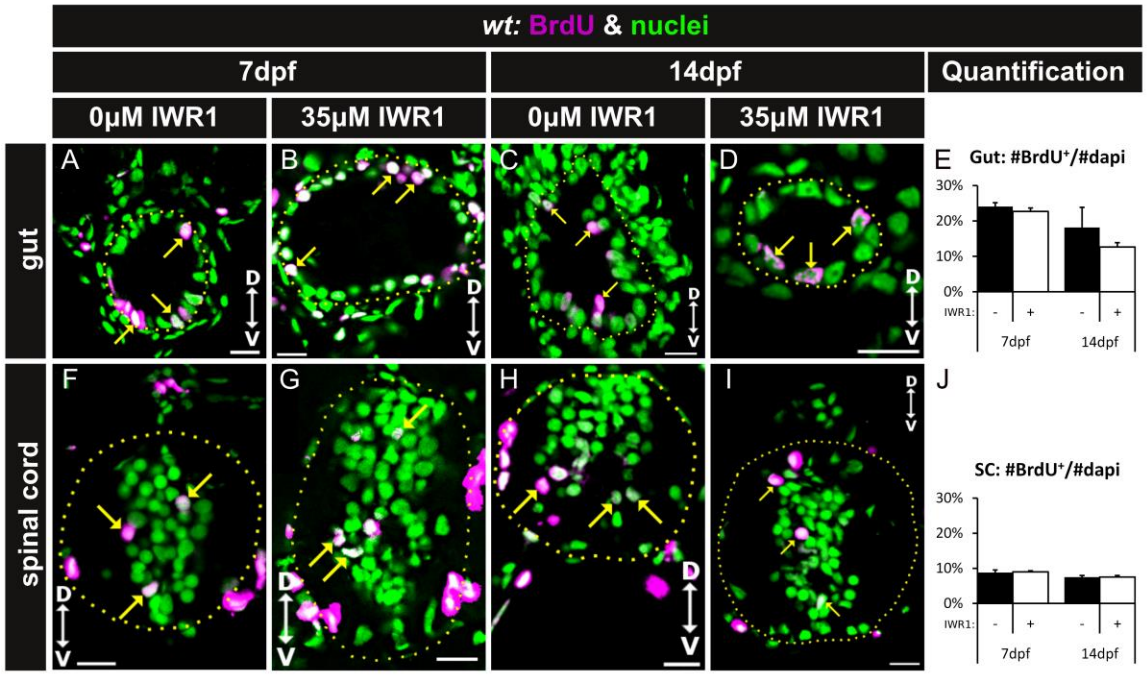

Figure 4.5: 35μM IWR1 does not affect endogenous rates of proliferation in uninjured animals. (A-D) BrdU incorporation in the gut. (A, C) controls; (B,D) with IWR1 for short pulse (B) or long pulse (D). (E) quantification of BrdU incorporation in the gut. (F-I) BrdU incorporation in the spinal cord. (F,H) controls; (G,I) with IWR1 for short pulse (G) or long pulse (I). (J) quantification of BrdU incorporation in the spinal cord. n=25 at each timepoint; error bars = SEM. Scalebar = 10μm.

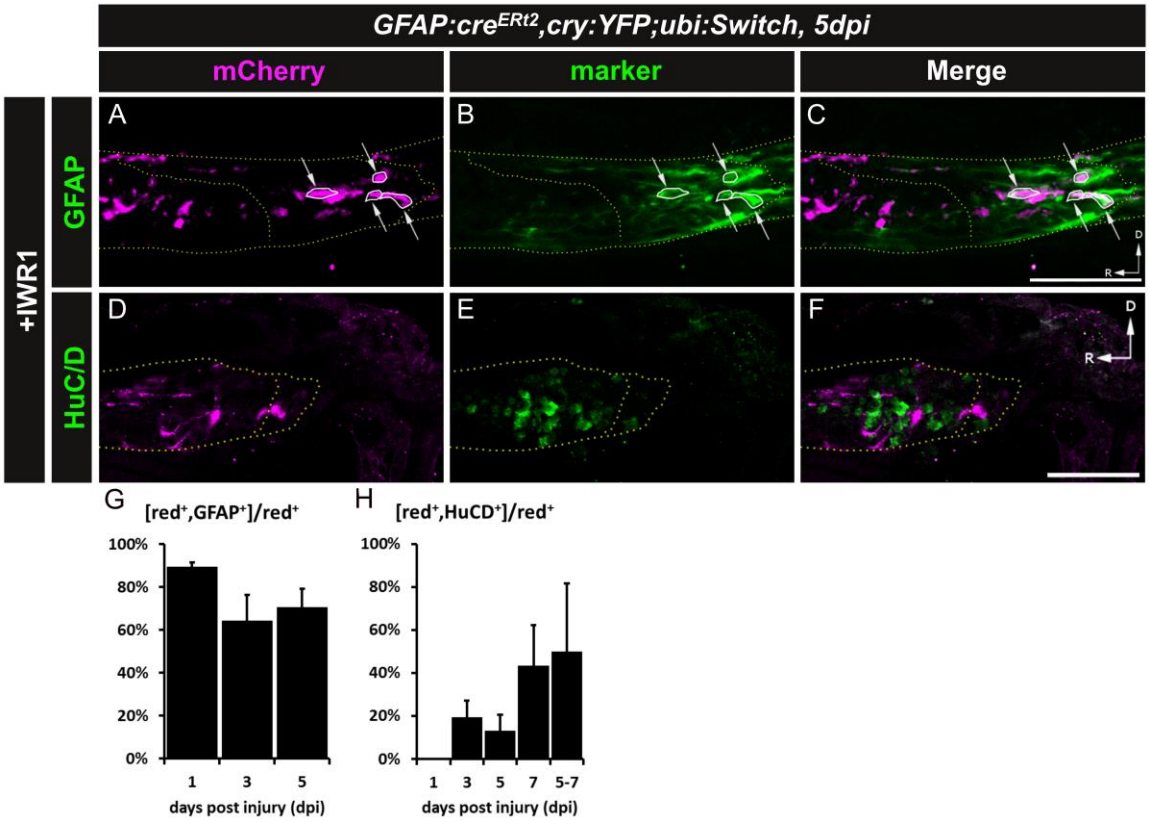

Figure 4.6: GFAPubi cells are responsive to Wnt/ß-catenin signaling. (A-C) more converted radial glia are observed in the blastema when Wnt/ß-catenin signaling is inhibited by the addition of IWR1 in the recovery media, compare with Figure 4.3. (J-L) Few converted neurons are observed in the blastema of IWR1-treated animals, compare with Figure 4.3. (G) Quantification of converted radial glia in the blastema + IWR1. (H) Quantification of converted neurons in the blastema + IWR1. n=5 at each timepont; error bars = SEM, scalebar = 50um.

CHAPTER 5

DISCUSSION

My studies originated from the following observations: that mammals lack the capacity for spinal cord regeneration while other vertebrates such as zebrafish retain that ability throughout their life, that Dbx1^{+} cells in the embryonic mouse spinal cord were multipotent but did not persist into adulthood, and that *dbx1a*$^{+}$ cells in zebrafish were neurogenic (Fogarty et al., 2005; Gribble et al., 2007, 2009). To unite these observations, I hypothesized that *dbx1a*$^{+}$ cells in the regenerative zebrafish were multipotent like their mammalian counterpart, and that they persisted beyond embryogenesis; that these cells were the key as to why zebrafish were regenerative in response to spinal cord injury (SCI) while mammals were not. Furthermore, I proposed that *dbx1a*$^{+}$ cells were able to contribute to the regeneration observed following SCI by reinitiating neurogenesis.

When I initially began my studies, an adult zebrafish model of spinal cord injury existed, but it was not without limitations: an average 6 week recovery time, a lack of tissue transparency, and reduced availability of genetic tools suggested that a companion model was necessary to address these shortcomings (Becker et al., 1997; Kim et al., 2000). Previously published evidence had shown that neural progenitor cells (NPCs) persisted in isolated niches of the zebrafish central nervous system (CNS) beyond embryogenesis, but there was minimal evidence of their presence or neurogenic potential in the spinal cord (Adolf et al., 2006; Kriegstein and Alvarez-Buylla, 2009; Reimer et al., 2008). The mechanisms regulating injury-induced neurogenic activity of

spinal NPCs were unknown, but regenerative studies following amputation suggested Wnt/β-catenin signaling as a candidate (Ito et al., 2007; Stoick-Cooper et al., 2007; Yokoyama et al., 2007). My research proved that the larval zebrafish was a suitable companion model for studying spinal cord regeneration; it also conclusively demonstrated that NPCs exist in the regenerative spinal cord, and that Wnt/β-catenin signaling moderated the neurogenic potential of NPCs following spinal cord injury.

Findings and conclusions

In Chapter 2, I established the larval zebrafish as a model for studying spinal cord regeneration following injury. I showed that the larval zebrafish injured at 5 days postfertilization (dpf) exhibited rapid repair, recovering sensory and motor function within 5-9 days postinjury (dpi) instead of the 6 weeks observed in the adult zebrafish. Moving to a larval model of SCI permits *in vivo* analysis of the regenerative process and use of genetic tools not otherwise available in the adult zebrafish. By severing the spinal cord at the level of the anal pore, I ensured that the injury was reproducible, and I also demonstrated that the larval zebrafish supports high throughput and large-scale analysis, with a trained technician able to injure 300 animals/hour. Initially developed for studying neurogenesis following spinal cord injury, this technique readily lends itself to examination of axonal regrowth or recovery of sensory function, thereby expanding the relevance and usefulness of this companion model of regeneration.

During mouse embryonic development, Dbx1 expression labels a multipotent population of cells that contribute to spinal neurons, astrocytes, and oligodendrocytes (Fogarty et al., 2005). Previous lab members have shown that the zebrafish ortholog *dbx1a* is expressed in the zebrafish spinal cord, that *dbx1a*$^{+}$ cells contribute to the V_0 interneuron population during development. They also established the transgenic line

Tg(dbx1a:eGFP) as an effective tool for studying *dbx1a*$^{+}$ cells (Gribble et al., 2007, 2009).

Since murine Dbx1^{+} cells are lost neonatally, I asked whether zebrafish *dbx1a*$^{+}$ cells persist beyond embryogenesis, based on the hypothesis that one of the key differences in the regenerative zebrafish and nonregenerative mouse was the persistence of a spinal neural progenitor population beyond embryogenesis. In Chapter 3, I showed that both *dbx1a* RNA and *dbx1a:eGFP* expression persists beyond embryogenesis. Additionally, I discovered that *dbx1a* expression identifies a subpopulation of neurogenic radial glia, suggesting that 1) *dbx1a*-expressing cells may have a role beyond embryogenesis and 2) that *dbx1a* expression may label a subpopulation of NPCs in the zebrafish spinal cord capable of responding to injury. NPCs persist as radial glia in the zebrafish brain (Johnson et al., 2014; Kizil et al., 2012; Kriegstein and Alvarez-Buylla, 2009), but have not been previously characterized in the spinal cord.

Using the transgenic line *Tg(dbx1a:eGFP)*, I showed that embryonic neurogenesis was complete by 5dpf, and confirmed that the response to SCI at 5dpf was regenerative rather than a continuation of development. Furthermore, I demonstrated that *dbx1a:eGFP*$^{+}$ cells proliferate in response to spinal transection, and are the source of most of the neurons born postinjury found in the blastema. Together, these data confirm that *dbx1a:eGFP*$^{+}$ NPCs are highly proliferative and neurogenic in response to spinal cord injury, and highlight the necessity for additional studies to elucidate the mechanism by which these cells are activated.

While *dbx1a* expression labels a subpopulation of NPCs, other subpopulations exist, including olig2^{+} and Sox2^{+} populations that contribute to motor- and Purkinje neurons, respectively (Kaslin et al., 2009; Reimer et al., 2008). Since NPCs persist in the zebrafish CNS as radial glia (Kriegstein and Alvarez-Buylla, 2009), in Chapter 4, we sought to generalize our analysis of NPCs by generating an inducible-Cre line under the

pan-glial promoter GFAP: *Tg(GFAP:Cre^{ERt2}, cry:YFP).* Crossing *Tg(GFAP:Cre^{ERt2}, cry:YFP)* to *Tg(ubi:loxP-GFP-stop-loxP-mCherry)* and screening for double-positive embryos ("*GFAPubi*") provided a way to permanently label zebrafish radial glia with temporal control.

Via immunohistochemistry, I showed that converted *GFAPubi* cells were detectable within 24 hours of induction with 4-hydroxytamoxifen, and that they were generally quiescent zrf1^{+} radial glia that minimally contributed to the spinal neuronal population during development. After SCI, however, they rapidly proliferated as radial glia, and in the 7dpi blastema, 43% of the neurons present were mCherry^{+}. Due to limitations in antibody availability, it will be difficult to characterize these converted cells further. However, utilization of other Cre-drivers expressed in neural progenitors in an injury model would be informative, such as *Nestin:Cre^{ERt2}*, *Sox2:Cre^{ERt2}*, and *Ascl1a:Cre^{ERt2}*, as would the use of a floxed-stop-marker transgenic line under the ubiquitin promoter such as *Tg(ubi:loxP-stop-loxP-GFP)* to facilitate colabeling studies (Kaslin et al., 2009; Lendahl et al., 1990; Parras et al., 2004).

As Wnt/β-catenin signaling has been demonstrated as necessary for regeneration after amputation (Stoick-Cooper et al., 2007; Yokoyama et al., 2007), I first used the Wnt reporter line *Tg(7xTCF:eGFP)* to determine if canonical Wnt signaling was expressed during spinal regeneration. I showed that in the blastema, Wnt/β-catenin signaling is expressed from 1-7dpi, and via immunohistochemistry, I also showed that a portion of GFP^{+} cells were radial glia that expressed the neural progenitor marker Sox3. Furthermore, I showed that these GFP^{+} NPCs made neurons after injury. Using the Wnt-inhibitor IWR1 to determine whether canonical Wnt signaling was required for spinal regeneration (Chen et al., 2009), I incubated injured larvae in IWR1 after surgery. This experiment showed a requirement for Wnt/β-catenin signaling from 2-5dpi, and that inhibition of Wnt/β-catenin signaling prevented converted radial glia from

differentiating as neurons. Together, these data suggest a mechanism by which spinal cord regeneration is governed, and identifies a new target of injury-induced neurogenic regulation for further study.

Significance

My work has answered several outstanding questions in the field of spinal cord regeneration; I have also developed several tools to help answer others. I have combined developmental biology, molecular biology, and microsurgical techniques to extend our knowledge of neurogenesis and regeneration in the zebrafish spinal cord.

When I began my studies, the cells responsible for postembryonic neurogenesis in the spinal cord were unknown. Examination of regenerating zebrafish spinal cord had revealed a glial progenitor population, but those *olig2*$^{+}$ cells only rarely make neurons (Park et al., 2007). I have shown that a neural progenitor niche exists outside the teleost brain: the spinal cord also harbors quiescent NPCs that can reinitiate neurogenesis beyond embryogenesis and in response to injury.

I have also shown that these NPCs are GFAP^{+} radial glial cells that are dependent upon canonical Wnt signaling for their proliferative and neurogenic responses to injury. These findings are significant: the NPC population in the regenerative zebrafish retina are also radial glial cells (Nagashima et al., 2013), which suggests that future investigation in regenerative neurogenesis following spinal cord injury should focus on local radial glia and their progeny.

Additionally, while the Goldman lab has shown a role for canonical Wnt signaling in the dedifferentiation of Müller glia following retina injury (Ramachandran et al., 2012), no other studies prior to my work have identified a role for Wnt/β-catenin signaling during stem-cell-based CNS regeneration. That spinal radial glia are unable to differentiate as neurons in the absence of canonical Wnt signaling raises the possibility

that any therapeutic intervention will have to include activation and modulation of Wnt/β-catenin signaling for functional and sensory recovery after spinal cord injury, and identifies an area ripe for further research.

I have established the larval zebrafish as a companion model to the adult for studying spinal cord regeneration, and have generated a new stable transgenic line whereby Cre recombinase is driven by the GFAP promoter to permanently label radial glia.

Previously, spinal cord regeneration analysis in the zebrafish was only possible in the adult (Becker et al., 1997), and therefore lacked access to potentially informative embryonic lethal mutations. Establishing an effective and robust model of spinal cord injury in the larval zebrafish now gives researchers a method providing access to genetic tools, *in vivo* and timelapse analyses to answer questions about axonal regeneration, functional recovery, and injury-induced neurogenesis.

With the ability to permanently label radial glia, I have created a new experimental paradigm whereby future researchers can ask further questions about the role of any signaling pathway implicated in injury-induced neurogenesis. Together, these tools can be exploited to further investigate why zebrafish can regenerate their spinal cord after injury, and mammals cannot.

Future directions

My work has confirmed that NPCs persist in the zebrafish spinal cord beyond embryogenesis, and has shown that their neurogenic potential is subject to Wnt/β-catenin signaling. However, my work has also identified additional questions for further study in the short term:

1) Are regenerated neurons integrated into existing circuitry and do they become physiologically active?

To determine whether regenerated neurons become physiologically active and integrated into spinal circuitry, we can use transgenic reporter lines such as *Tg(HuC:GCaMP6f-GFP)* or *Tg(HuC: loxP-stop-loxP-GCaMP6f-GFP)* to study neuronal calcium signaling. By imaging injured animals to detect action potentials in regenerating neurons, we will be able to determine to what extent neurogenic repair contributes to functional and sensory recovery.

2) Does the inhibition of canonical Wnt signaling block functional or sensory recovery?

Wnt/β-catenin signaling has been implicated in virtually all models of regeneration examined to date, suggesting that this signaling pathway may be the key to unlocking the regenerative potential in mammals. I and others have shown the necessity of canonical Wnt signaling in injury-induced proliferation of NPCs and their differentiation as neurons. However, it is not known whether Wnt/β-catenin signaling plays a role in re-establishing lost neuronal connectivity. Treating injured *Tg(HuC:GCaMP6f)* fish with Wnt inhibitor IWR1 will help answer this question.

3) What cell types are secreting Wnt after SCI?

As Wnt is a secreted ligand, knowing which cell types are expressing Wnt after SCI may reveal additional therapeutic targets in the nonregenerative mammalian spinal cord. Promising candidates include microglia and macrophages; these are actively being investigated by other groups using gene knock-out lines to prevent the generation of these cell types.

4) What downstream targets of Wnt/β-catenin signaling are involved in injury-induced spinal neurogenesis?

Lastly, identifying downstream targets of injury-induced Wnt signaling as possible initiators of neurogenesis will provide additional subjects of research for those seeking to ameliorate the effects of SCI in humans. Possible candidates include the

proneural gene *ascl1a* and zinc-finger transcription factor *sp5-like* (Guo et al., 2011; Ramachandran et al., 2011; Weidinger et al., 2005)

If we consider the larger goal of functional recovery following SCI in humans, in the light of my findings, major questions become apparent:

- Why is the inflammatory response permissive to regeneration in zebrafish but inhibitory in humans?
- What are the target cells in the human spinal cord for neurogenic reactivation following injury?

A *role for macrophages in spinal cord regeneration?*

In both zebrafish and mammals, SCI triggers an immediate inflammatory response: macrophages are recruited to the injury site, targeting cellular debris and damaged axons (Hui et al., 2010, 2014; Kigerl et al., 2009). Intriguingly, two distinct subsets of macrophages exist in mammals: the pro-inflammatory M1 macrophages, and the anti-inflammatory M2 (Hayakawa et al., 2014). M1 macrophages are the first to respond to SCI in the mouse, and maintain high levels of expression at the site of injury for at least 1 month postinjury. M2 macrophages are not present at the injury site until 3dpi, and are detectable only until 7dpi (Kigerl et al., 2009).

In the zebrafish, macrophages are characterized by expression of *mpeg1*. Zebrafish macrophage subclasses may also exist, but a lack of markers prevent their categorization (van der Vaart et al., 2012). After SCI, both pro- and anti-inflammatory genes associated with increased macrophage activity are upregulated in the zebrafish spinal cord (Hui et al., 2014).

Following caudal fin amputation in *Tg(mpeg1:mCherry)* fish, macrophages accumulate in newly regenerated tissue by 3-4 days postamputation (dpa) (Petrie et al., 2014); canonical Wnt ligand wnt10a is also present in the newly regenerated caudal fin at

3dpa (Stoick-Cooper et al., 2007). By expressing nitroreductase (NTR) under the *mpeg1* promoter, the Moon group was able to use the pro-drug metronidazole and *Tg(mpeg1:NTR)* to cytotoxically kill macrophages. To examine the role of macrophages on the caudal fin regeneration, *Tg(mpeg1:NTR)* was crossed to the Wnt reporter line *Tg(7xTCF:mCherry)* and the double transgenic F1s were used for analysis. In the absence of macrophages, fewer Wnt-reporter expressing cells ($mCherry^{+}$) were detectable in the blastema, and a significant reduction in blastemal proliferation was observed at 3dpa, indicating a role for macrophages in both injury-induced Wnt expression and proliferation (Petrie et al., 2014).

I propose that M2 macrophages are the source of canonical Wnt ligands in the regenerative spinal blastema. In an *in vitro* model of chronic liver disease, macrophage engulfment of hepatic debris triggered Wnt-3a and -7a expression; however, it is not known whether these Wnt ligands were secreted by M1 or M2 macrophages (Boulter et al., 2012). Additional *in vitro* analysis of macrophages co-cultured with colorectal adenocarcinoma cells revealed that M2 but not M1 macrophages expressed canonical Wnts -1 and -3a (Cosín-Roger et al., 2013).

Injuring larval *Tg(mpeg1:NTR;7xTCF:mCherry)* fish pretreated with NTR would reveal whether macrophages were involved in spinal cord regeneration, and whether they mediate Wnt signaling in the regenerating spinal cord. Examining the regenerative response of injured *Tg(mpeg1:NTR;GFAP:Cre^{ERt2};Ubi:switch)* would elucidate the role of macrophages in governing the radial glial contribution to injury-induced neurogenesis. It would also be informative to examine sensory and functional recovery following spinal cord injury in a mouse expressing the MaFIA transgene, which supports conditional and reversible macrophage ablation (Burnett, 2004). Alternatively, postinjury adoptive transfer of M2 macrophages into adult mice would conclusively demonstrate the importance of macrophages to spinal regeneration (Parsa et al., 2012).

Are there quiescent NPCs in the mammalian spinal cord?

The NPCs of the zebrafish spinal cord are GFAP$^+$ radial glia cells (RG) that persist as a relatively quiescent population in the absence of injury (unpublished data). However, in the developing mammalian spinal cord, once multipotent progenitor cells transition into RG, they cease cell division and terminally differentiate as astrocytes (Barry and McDermott, 2005; Pakan and McDermott, 2014). However, spinal ependymomas (glial tumors found in both children and adults) are believed to arise from rare multipotent RG-like cells that maintain the ability to self-renew (Taylor et al., 2005). High-risk ependymomas express elevated levels of nestin, a neural progenitor marker (Milde et al., 2012) and tend to have increased ErbB2 expression (Garcia and Gutmann, 2014). Together, these findings indicate that not all mammalian spinal NPCs terminally differentiate as astrocytes. Furthermore, these data suggest that those few spinal NPCs that persist beyond embryogenesis preferentially support a neuronal instead of a glial fate.

I hypothesize that mammalian spinal NPCs might be identified by co-expression of GFAP, nestin, and ErbB2, and that these cells are therapeutic targets for neurogenic activation following injury. Once identified and characterized, these cells could then be isolated for adoptive transfer into injured animals. In a companion study, zebrafish could be injured and treated with ErbB2 inhibitors SKLB1206, Trastuzumab, or Pertuzumab to confirm whether ErbB2 expression is implicated in spinal regeneration (Pan et al., 2012; Tebbutt et al., 2013).

Summary

The results described in this dissertation advance the field of spinal cord regeneration by confirming the presence of NPCs in the postembryonic spinal cord that exhibit injury-induced neurogenesis. Initially focused on *dbx1a*$^+$ NPCs, I have been able

to generalize our findings to a subpopulation of GFAP$^+$ radial glial NPCs, thereby increasing the relevance of this work. I have established a new transgenic line capable of permanently labeling a population of radial glia and have provided preliminary data demonstrating that Wnt/β-catenin signaling is required for the neurogenic response after SCI, thereby laying the groundwork for further analysis of spinal cord regeneration. Finally, I have identified multiple opportunities for future efforts that will extend the findings of this research and open new areas of investigation.

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